Cardiomyocyte Polarity and Embryonic Heart Development: Role of Rac1

Carmen Leung  
*The University of Western Ontario*

Supervisor  
Qingping Feng  
*The University of Western Ontario*

Graduate Program in Physiology and Pharmacology  
A thesis submitted in partial fulfillment of the requirements for the degree in Doctor of Philosophy  
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Cardiomyocyte Polarity and Embryonic Heart Development:  
Role of Rac1

(Thesis format: Integrated Article)

by

Carmen Leung

Graduate Program in Physiology and Pharmacology

A thesis submitted in partial fulfillment  
of the requirements for the degree of  
Doctor of Philosophy

The School of Graduate and Postdoctoral Studies  
The University of Western Ontario  
London, Ontario, Canada

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Abstract

Congenital heart defects (CHDs) are the most common human birth defect and the leading cause of death from a birth defect in the first year of life. Thus, a further understanding of the mechanisms underlying CHDs, which could lead to improved diagnosis and treatment, is crucial. The small GTPase, Rac1, acts as a pleiotropic effector of numerous cellular processes; however, little is known about its role in embryonic heart development. The aim of this thesis was to investigate the role of Rac1 signaling in cardiac development. Using the Cre/loxP system, mouse models with an anterior second heart field (SHF) or ventricular myocardium specific deletion of Rac1 were generated. I demonstrated that mice with a SHF Rac1 deficiency displayed a spectrum of CHDs including bifid cardiac apex and septal defects. These Rac1 deficient cardiomyocytes failed to undergo polarization. In addition, migration and lamellipodia formation was disrupted in Rac1 deficient cardiomyocytes. Decreased SHF Rac1 signaling led to increased apoptosis and downregulation of several cardiac developmental transcription factors. Furthermore, my data showed that organization of anterior SHF progenitors was disrupted, leading to a spectrum of outflow tract (OFT) defects in Rac1 deficient mice. Cardiomyocytes with a Rac1 deficiency failed to polarize and undergo myocardialization, causing misalignment of the OFT. At P0, Rac1 deficient aortic valves displayed defects in maturation and remodeling and intrauterine echocardiography performed at E18.5 showed severe aortic valve regurgitation. Finally, I showed that a Rac1 deficiency in the ventricular myocardium resulted in ventricular noncompaction and defective trabecular development. The myocardium proliferation rate was significantly decreased and expression of the planar cell polarity protein, Scrib, was reduced. Actin polymerization was severely disrupted and cardiomyocytes failed to polarize in E18.5 Rac1 deficient ventricular myocardium. In conclusion, my thesis is the first to show
that Rac1 signaling regulates several pathways, including proliferation, cell survival, gene expression and actin polymerization during embryonic heart development. Through these mechanisms, Rac1 is a critical regulator of cardiomyocyte polarity, septal and OFT development and formation of the ventricular myocardium.

**Keywords:** Rac1, cardiomyocyte polarity, congenital heart defects, heart development, cell organization, migration, outflow tract, second heart field
Co-Authorship Statement

The studies outlined in Chapter 2-4 were performed by Carmen Leung in the laboratory of Dr. Qingping Feng, with the assistance of co-authors as listed below.

Dr. Qingping Feng contributed to experimental design, data interpretation and manuscript preparation for all experiments. Dr. Thomas A. Drysdale contributed to manuscript preparation for Chapter 3. In addition, Dr. Sharon Lu assisted with the scientific training and troubleshooting in all experiments. Ms. Murong Liu performed animal caretaking, breeding and genotyping.

Chapter 2: Dr. Sharon Lu assisted with the western blot analysis (Figure 2.1), imaging of the bifid cardiac apex (Figure 2.2 A, B), microdissection of E12.5 right ventricles for real-time PCR (Table 2.4) and explant culture (Figure 2.5) and primary cardiomyocyte cultures (Figure 2.5 and Figure 2.6). Ms. Murong Liu also helped with the primary cardiomyocyte cultures (Figure 2.5 and Figure 2.6).

Chapter 3: Ms. Mella Kim worked as a high school co-op student in the lab and helped with tissue processing and histology. Dr. Yin Liu assisted with intrauterine echocardiography (Figure 3.9).

Chapter 4: The Nkx2.5-Cre mouse was a generous gift from Dr. Chi-chung Hui. Dr. Sharon Lu helped with western blot analysis (Figure 4.1) and imaging of the bifid cardiac apex (Figure 4.3A).
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I am also thankful for my lab colleagues, who have made my time as a graduate student enjoyable and full of great memories. Most importantly, I would like to thank Dr. Sharon Lu who taught me almost everything I know in lab from the day I first started. Sharon is the most knowledgeable lab manager I know and she is the glue that holds the Feng lab together. I would like to thank her for all of her valuable troubleshooting advice and assistance with all of my projects. Many of my experiments would not have been possible without her. I would also like to thank Murong Liu for her help with mouse handling and genotyping. Murong’s ability to keep track and take care of the many genetic mouse lines in the Feng lab was impeccable. Thanks to the many volunteers, summer students, high school co-op and 4th year thesis students who have been very helpful in lab: Jess Ngan, Derek Little, Mella Kim, Simran Aulakh, Tana Saiyin and Cameron Webb. Graduate school life would not have been as enjoyable without the many past and present members of the Feng lab
throughout the years. Thank you especially to Lamis Hammoud, who let me tag along and learn from her when I first started in the Feng lab. Thanks also go out to Paul Arnold, Yin Liu, Lily Xiang, Ting Zhang, Hoda Moazzen, Yan Wu, Lili Zhang, Joey Qin, Dan Secor, Katherine Lee, Anish Engineer and Jess Blom for their camaraderie and friendship. Many of the great memories I have from grad school are from these friendships. Thank you also to the many other great people on the 2nd floor of MSB and in Phys/Pharm who have enriched my grad school experience. I would also like to thank all of my friends outside of graduate school, especially my former roommates Deanna Vertesi and Vincci Siu, who have always been supportive and encouraging.

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<th>Full Name</th>
</tr>
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<tbody>
<tr>
<td>αMHC</td>
<td>α-myosin heavy chain</td>
</tr>
<tr>
<td>ANP</td>
<td>atrial natriuretic peptide</td>
</tr>
<tr>
<td>Ao</td>
<td>aorta</td>
</tr>
<tr>
<td>API</td>
<td>activator protein-1</td>
</tr>
<tr>
<td>aPKC</td>
<td>atypical protein kinase C</td>
</tr>
<tr>
<td>Arp2/3</td>
<td>actin-related-protein-2/3</td>
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<tr>
<td>ASD</td>
<td>atrial septal defect</td>
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<tr>
<td>AV</td>
<td>atrioventricular</td>
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<tr>
<td>AVC</td>
<td>atrioventricular canal</td>
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<tr>
<td>AVE</td>
<td>anterior visceral endoderm</td>
</tr>
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<td>bHLH</td>
<td>basic helix-loop-helix</td>
</tr>
<tr>
<td>BMP</td>
<td>bone morphogenetic protein</td>
</tr>
<tr>
<td>Cdc42</td>
<td>cell division control protein 42</td>
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<tr>
<td>CHD</td>
<td>congenital heart defect</td>
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<tr>
<td>Cx</td>
<td>connexin</td>
</tr>
<tr>
<td>Daam1</td>
<td>DVL-associated activator of morphogenesis-1</td>
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<tr>
<td>DGS</td>
<td>DiGeorge Syndrome</td>
</tr>
<tr>
<td>DORV</td>
<td>double outlet right ventricle</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>Dvl</td>
<td>Dishevelled</td>
</tr>
<tr>
<td>E</td>
<td>embryonic day</td>
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<td>ECM</td>
<td>extracellular matrix</td>
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<tr>
<td>FGF</td>
<td>fibroblast growth factor</td>
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<tr>
<td>FHF</td>
<td>first heart field</td>
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<tr>
<td>GAP</td>
<td>GTPase activating protein</td>
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<td>GDI</td>
<td>guanine dissociation inhibitor</td>
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<td>GDP</td>
<td>guanine diphosphate</td>
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<td>GEF</td>
<td>guanine nucleotide exchange factor</td>
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<td>guanine triphosphate</td>
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<td>Hh</td>
<td>Hedgehog</td>
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<td>HOS</td>
<td>Holt-Oram Syndrome</td>
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<tr>
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<td>IQ motif containing GPTase activating protein</td>
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<td>Isl1</td>
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<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
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<tr>
<td>LA</td>
<td>left atrium</td>
</tr>
<tr>
<td>LV</td>
<td>left ventricle</td>
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<td>LVNC</td>
<td>left ventricular non-compaction cardiomyopathy</td>
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<tr>
<td>MAPK</td>
<td>p38 mitogen-activated protein kinase</td>
</tr>
<tr>
<td>Mef2c</td>
<td>myocyte-specific enhancing factor 2</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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</tr>
<tr>
<td>MET</td>
<td>mesenchymal-to-epithelial transition</td>
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<td>NF-κB</td>
<td>nuclear factor kappa-light-chain-enhancer of activated B cells</td>
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<td>OFT</td>
<td>outflow tract</td>
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<td>PA</td>
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<td>PAK</td>
<td>p21 activating kinases</td>
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<td>PAR</td>
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<tr>
<td>PI3K</td>
<td>phosphoinositide 3-kinase</td>
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<tr>
<td>PLXN</td>
<td>plexin</td>
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<td>PTA</td>
<td>persistent truncus arteriosus</td>
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<td>right atrium</td>
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<td>Rac1</td>
<td>Ras-related C3 botulinum toxin substrate 1</td>
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<td>RhoA</td>
<td>Ras homolog gene family, member A</td>
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<td>RV</td>
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<td>SHF</td>
<td>second heart field</td>
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<tr>
<td>Sema</td>
<td>semaphorin</td>
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<td>SRF</td>
<td>serum response factor</td>
</tr>
<tr>
<td>Tbx</td>
<td>T-box</td>
</tr>
<tr>
<td>TOF</td>
<td>Tetralogy of Fallot</td>
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<tr>
<td>Acronym</td>
<td>Description</td>
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<td>-------------</td>
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<tr>
<td>VSD</td>
<td>ventricular septal defect</td>
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<tr>
<td>WAVE</td>
<td>Wiskott-Aldrich syndrome protein with a V-domain</td>
</tr>
<tr>
<td>WGA</td>
<td>wheat germ agglutinin</td>
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1 Chapter 1 Introduction

1.1 Heart Development

The heart is the first organ to form and function in all vertebrates and is critical in ensuring proper nutrient and oxygen supply to the developing embryo. Heart development is a complex, highly regulated and multi-stepped process of myogenesis and morphogenesis where populations of progenitor cells must merge in a temporally and spatially coordinated fashion. The heart develops from three distinct populations of cells: the first heart field (FHF), second heart field (SHF) and neural crest cells. FHF progenitors originate from the anterior lateral plate mesoderm and form two lateral heart fields along the midline. The two heart fields will then coalesce and form a crescent shape in the anterior embryo by E7.5 in the mouse. At approximately E8.0, the FHF cells merge along the ventral midline to form the primitive beating heart tube. The heart tube is made up of an interior layer of endocardial cells and an outer layer of myocardial cells with extracellular matrix in between the two layers. This tube undergoes rightward looping by E9.0 and begins early chamber morphogenesis.\(^1\) SHF cells originate from pharyngeal mesoderm and lie anteriorly and medially to the FHF cardiac crescent. As the heart tube forms, SHF cells migrate into the midline and lie dorsal to the heart tube. Upon rightward looping of the heart tube, SHF cells cross the pharyngeal mesoderm and are added progressively into the arterial and venous poles of the heart tube, differentiating into myocardial, endocardial and smooth muscle cells of the outflow tract (OFT), future right ventricle (RV), atria and interventricular septum.\(^2-5\) As well, following looping morphogenesis, endocardial cushions will begin to form within the OFT and the
atrioventricular canal (AVC), which will later help septate the common OFT and divide the heart into four chambers (Figure 1.1).\textsuperscript{6} SHF progenitors have elevated rates of proliferation and differentiation delay with respect to the FHF cells that contribute to the heart tube.\textsuperscript{7} The SHF also expresses distinct genes that distinguish it from the FHF including the growth factors Fgf10 and Fgf8 and the transcription factors Tbx1, Islet1 (Isl1) and Mef2c.\textsuperscript{8-13} Compared to the SHF, the FHF is difficult to locate due to the lack of distinguishing markers.\textsuperscript{2} Along with the SHF progenitors, neural crest cells migrate from the neural tube and contribute to the remodeling and septation of the OFT, which will become the aorta and pulmonary artery of the heart.\textsuperscript{14} By E15.5, the embryonic heart structure is mostly complete except for the semilunar andatrioventricular valves, which will continue to undergo remodeling and maturation after birth.\textsuperscript{15}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{Heart development in the mouse.}
\end{figure}

Oblique views (top panels) and frontal views (lower panels) of cardiac progenitor cells in the embryonic mouse. FHF progenitors form a cardiac crescent by E7.5 and will merge to
form the primary heart tube by E8.0. SHF progenitors will migrate into the heart tube to elongate the arterial and venous poles. After rightward looping of the heart tube, cardiac neural crest (CNC) cells will also migrate into the OFT to contribute to cardiac development. V, ventricle; A, atrium; CT, conotruncus; RA, right atrium; LA, left atrium; AS, aortic sac; RV, right ventricle; LV, left ventricle; AVV, atrioventricular valves; Ao, aorta; PA, pulmonary artery; DA, ductus arteriosus; RSCA, right subclavian artery; RCA, right carotid artery; LCA, left carotid artery; LSCA, left subclavian artery.

Figure adapted with modifications from Srivastava, D. Cell. 2006;126:1037-1048.

1.1.1 Second Heart Field

The existence of extra-cardiac progenitor cells that contribute to later formation of the OFT myocardium was first suggested in 1977 by de la Cruz et al. The question of where the OFT myocardium originated from remained unclear until 2001 when three different groups published reports of a separate population of cells that contribute to the initial heart tube in both chick and mice. In one initial chick study, a group of cells called “secondary heart field” was found adjacent and posterior to the OFT region while in the other chick study, the newly discovered group of cells was termed “anterior heart field” and encompassed a larger region of the cranial pharyngeal mesoderm extending into the pharyngeal arches. In mice, Kelly et al. discovered a population of anterior heart field cells, which expressed lacZ under the control of Fgf10 regulatory elements in the splanchnic and pharyngeal mesoderm, contributing to the RV and myocardial cells at the arterial pole of the heart tube. A later study on the LIM homeodomain transcription factor Isl1 found this region of the murine anterior heart field to be actually more extensive, extending more posteriorly and contributing to elongation of both the arterial
and venous pole of the heart tube. Thus, the SHF is subdivided into an anterior and posterior domain where the anterior region is marked by the Fgf10-lacZ transgene while Isl1 marks both domains.\textsuperscript{9,13}

A complex network of signaling pathways and transcription factors regulate the SHF progenitor cell population in the dorsal pericardial wall as it proliferates and progressively differentiates in the OFT. One example is retinoic acid signaling, which plays a role in defining the posterior boundary of the SHF progenitor cells and later on in differentiation of the distal OFT progenitor cells.\textsuperscript{19} Fibroblast growth factors (Fgf), including Fgf3, Fgf8 and Fgf10, play a part in regulating the high rate of SHF progenitor cell proliferation in the dorsal pericardial wall.\textsuperscript{20} Upstream of this, Wnt/\(\beta\)-catenin and Notch signaling regulate the expression of FGF ligands.\textsuperscript{21} Studies have shown that conditional loss of Fgf, Wnt/\(\beta\)-catenin and Notch signaling in the SHF leads to a shortened OFT and OFT defects.\textsuperscript{20,22,23} In addition, Hedgehog (Hh) signaling has been demonstrated to be critical for SHF progenitor cell proliferation by acting through the transcription factor, Tbx5.\textsuperscript{24} Numerous other transcription factors play critical roles in the SHF including Isl, Tbx1, Six1, and Eya1, which regulate FGF expression, proliferation and cell survival of progenitor cells.\textsuperscript{25-27} As SHF cells migrate into the heart tube, proliferation decreases and there is an onset of myocardial differentiation. Bone morphogenetic protein (BMP) signaling at the distal OFT promotes differentiation of SHF cells by antagonizing FGF signaling.\textsuperscript{28} Together with BMP signaling, SHF progenitors residing proximal to the heart tube will initiate expression of Gata4, Nkx2.5, Tbx20, Mef2c and Hand2 to activate the myocardial gene expression program.\textsuperscript{18}
Mef2c is a member of the myocyte-specific enhancing factor 2 (Mef2) family of MADS-box transcription factors, which has been shown to be critical for cardiac development. There are four Mef2 genes in vertebrates: Mef2a, Mef2b, Mef2c and Mef2d, which bind a conserved A-T rich DNA control region associated with cardiac, skeletal and smooth muscle lineages. Mef2c is one of the earliest markers of the cardiac lineage at E7.5 and precedes expression of α-cardiac actin, a sarcomeric protein. Strong Mef2c expression in the developing heart continues from E7.5 to E11.5 and then begins to decline. Mef2c<sup>−/−</sup> mice have a SHF phenotype where the heart fails to loop, the OFT has gross abnormalities and the RV does not develop. Dodou et al. discovered an enhancer and promoter from the Mef2c gene that was expressed exclusively in the anterior heart field during cardiac development. Subsequent fate mapping studies using Cre expression under the control of the Mef2c anterior heart field promoter and enhancer (Mef2c-AHF-Cre) show that lacZ expressing cells mark progenitors of the SHF that contribute to formation of the RV, ventricular septum and the endothelial and myocardial cells of the OFT. Thus, the Mef2c-AHF-Cre is the first transgenic mouse line with Cre activity restricted to the anterior heart field and its derivatives, making it an excellent tool for genetic studies in the SHF. Hence, this Mef2c-AHF-Cre transgenic mouse line was used to study the SHF in Study 1 and 2.

1.1.2 Neural Crest Cells

Neural crest cells are a multipotent population that migrates from the dorsal neural tube during embryonic development to contribute to numerous tissue types including the peripheral nervous system, melanocytes and tissues of the head and neck. Early transplantation and cell labeling experiments in avian models identified a specific
region of the dorsal neural tube that contributed to parts of the heart and associated vessels. Following these observations, subsequent chick studies showed that ablation of the neural crests in the cranial region before the cells became migratory led to a failure of the common OFT to divide into aorta and pulmonary trunk, abnormal patterning of the aortic arch and great arteries, OFT alignment defects and VSDs. Since these seminal discoveries in the chick embryo, this population of neural crest cells has been termed “cardiac neural crest” due to their importance in heart development. Subsequent studies in transgenic mouse models using neural crest promoters have confirmed the importance of the cardiac neural crest in cardiovascular development that was first observed in avian models. Cardiac neural crest cells delaminate from the neural tube at approximately E8.5 in the mouse, migrate and pause in the circumpharyngeal ridge before migrating into and populating pharyngeal arches three, four and six as each arch forms. These neural crest cells play a critical role in repatterning of the pharyngeal arches to become the great arteries of the thorax, including common carotid, aortic arch and ductus arteriosus. Additionally, a subpopulation of cardiac neural crests from the pharyngeal arches will migrate into the OFT cushions at approximately E9.5 to form the aorticopulmonary septation complex, which will divide the common outflow tract into the aorta and pulmonary trunk. Development and remodeling of the semilunar valves (aortic and pulmonary valves) also rely on tissue-tissue interactions between the cardiac neural crest and the SHF cells. As neural crest cells migrate into the developing heart, they are closely juxtaposed to migrating SHF progenitors in the pharyngeal mesenchyme where they are able to influence each other through various signaling mechanisms. Defects in SHF progenitors or the cardiac neural crest cell population can have secondary effects on
the other cell type, supporting an intricate relationship between these two cell types in cardiac development.\textsuperscript{43-45}

1.1.3 Development of the Septated Heart

From E8.25 to E10.5 in the mouse, the heart tube will undergo rightward looping, which is the first event displaying left-right asymmetry during embryonic development. As looping proceeds, the RV and left ventricle (LV) become arranged side by side and connected to the common OFT. The distance between the atrial and venous poles shorten as they converge and the ventricular bend will shift caudally. In the late stages of looping, the proximal OFT will shift to the left and the heart tube will undergo ballooning of the atrial and ventricular regions. The OFT undergoes remodeling and the aortic valve becomes wedged between the AV valves.\textsuperscript{46} As looping morphogenesis occurs, SHF progenitors are concurrently being added into the heart tube to subsequently give rise to a majority of the RV, interventricular septum and OFT. A failure of SHF cells to incorporate into the heart tube can interrupt cardiac looping.\textsuperscript{13} As the heart completes looping, it will undergo a septation process through growth of myocardium and valve development and remodeling to form a four-chambered heart.

Separation of the atria from the ventricles occurs through development of AV valves (mitral and tricuspid valves), which originate from AV endocardial cushions formed at E9.0. The AV endocardial cells undergo a process of EMT and the cushions will remodel and gradually maturate to form thin, valve leaflets. During this process of remodeling and maturation, the mesenchymal cells will proliferate and extracellular matrix (ECM) deposition will extend the cushions to form the valve leaflets. Development of the AV valves is dependent on numerous signaling factors secreted by
the myocardium underlying the cushions, including BMP, TGFβ and Vegf. These factors will promote EMT of the endocardial cushion. In addition, the endocardial cells also express receptors for Nfatc1 and Vegf, which are critical for valve development. The semilunar valves (aortic and pulmonary valves) arise from the conal and intercalated cushions in the OFT. The right and left leaflets of the semilunar valves are formed from the conal cushions. The intercalated cushions will develop into the posterior aortic and anterior pulmonic leaflets. Similar to development of the AV valves, OFT conal cushions undergo a process of EMT to give rise to mesenchymal cells that proliferate, followed by matrix deposition, elongation and remodeling/thinning of the valve leaflets. Development of the semilunar valves requires similar signaling pathways, transcription factors, epigenetics and morphogenetic events as those required for AV valve development.

Besides development of cardiac valves to septate the heart, the atrial and ventricular chambers must also undergo septation to divide it into their left and right counterparts. The muscular part of the interventricular septum is formed between the two future ventricles from ballooning of the apical components. This muscular septum will continue to grow superiorly towards the AV cushions, leaving an opening at the AV canal between the two ventricular chambers. This opening will be closed by the membranous part of the interventricular septum that develops from the AV cushions. Tbx5 has been shown to be essential for determining LV identity and the interventricular boundary. Overexpression of Tbx5 in mice causes expansion of the LV and loss of the interventricular septum. Compared to ventricular septation, atrial septation is a more complex process. A muscular septum, called the primary atrial septum, begins to grow at
E9.5 from the atrial roof towards the AV cushions. The edge of the primary septum contains a mesenchymal cap. Growth of the primary septum will partially septate the atrial chamber, leaving an opening called the ostium primum that allows shunting of blood between the two chambers. The mesenchymal cap will fuse with the AV cushion and the dorsal mesenchymal protrusion (DMP), which is mesenchymal tissue from the dorsal mesocardium, to close the ostium primum. As this occurs, apoptosis occurs on the anterior side of the primary septum to create a second opening called the ostium secundum, which is later closed by the muscular secondary atrial septum. Many signaling pathways and transcription factors are critical to atrial septum formation including BMP signaling and the GATA and TBX family of transcription factors.

Septation of the heart is dependent on interactions between different cardiac cell lineages, including the SHF and cardiac neural crest cells. Besides complex signaling mechanisms that exist within each lineage, progenitors of each lineage interact with other cells to coordinate the septation process. Lineage tracing studies show that a large part of the interventricular septum is derived from SHF progenitors. The DMP that contributes to atrial septum is also derived from SHF progenitors. Many studies have demonstrated that disruption of several different signaling pathways in the SHF result in defective septation of the heart. For example, disruption of calcineurin/Nfatc1 signaling in the SHF led to increased apoptosis, regression of OFT cushions and a subsequent absence of semilunar valve formation. Hedgehog and BMP signaling is essential for development of the DMP for atrial septation. In addition, deletion of the transcription factor Hand2 or disruption of BMP4 signaling in SHF progenitors lead to defects in interventricular septum formation in mouse embryos.
Cardiac neural crest cells have roles in OFT septation and development of the semilunar valves as well, with little to no contribution to AV valve development. These cells are crucial for remodeling and maturation of the semilunar valves and formation of the aorticopulmonary complex to septate the OFT. Cardiac neural crest cells will also interact with surrounding tissues, such as the SHF-derived OFT myocardium through semaphorin signaling to migrate into the developing OFT. Specifically, the semaphorin Sema3c is expressed in the SHF-derived OFT myocardium and signals to PLXNA2 receptors expressed on migrating cardiac neural crest cells.

1.1.4 Ventricular Myocardium Development

Maturation of the ventricular chamber myocardium is essential for increased cardiac output as an embryo grows and develops. The ventricles grow and mature through a process of proliferation and differentiation to form trabeculation and the compact myocardial layer. In general, cardiac chamber maturation can be divided into three processes: formation of trabeculae, formation of conduction system and thickening of the compact myocardium. Trabecular formation begins in the mouse at approximately E9.5 where cardiomyocytes grow to form protrusions of muscular ridges that become lined by invaginating endocardial cells. As these ridges form, the trabecular projections grow and spread radially to form a network of trabeculation. This formation of trabeculation facilitates oxygen and nutrient exchange in the heart and enhances force generation during contraction. The trabeculae then undergo a process of remodeling or compaction where growth stops and the spaces between trabeculae become capillaries. The bases of trabeculae thicken and collapse into the myocardial wall. By E14.5, a mature trabecular network is formed, along with formation of a thick, compact
ventricular myocardium. A gradient of decreasing proliferation and increasing differentiation exists from the compact myocardium zone towards the trabecular zone. Proliferation of the compact myocardium is concomitant with remodeling of the trabeculation. Signaling from the endocardium and epicardium to myocardial cells have been demonstrated to be critical for trabeculation and ventricular development. For example, neuregulin 1, produced by endocardial cells, act on receptors in the myocardium to regulate trabecular development. The cardiac jelly between the endocardium and myocardium plays a crucial role in transmission of signals between these two cell types. In addition, FGF signaling from the epicardium to myocardial cells has been demonstrated to be a critical regulator of ventricular wall development. Interestingly, endocardial-derived signals seem to regulate trabecular development while epicardial-derived signals seem to mediate compact myocardium development. Numerous signaling pathways have been implicated in maturation of the cardiac ventricular chambers including Notch, ephrin, BMP, FGF, retinoic acid and ECM signaling pathways. In addition, biomechanical forces such as shear stress can influence signaling pathways that regulate chamber maturation. However, the relationship between these physical forces, gene expression and chamber development is not well understood.

1.1.5 Outflow Tract Development

In early development, the cardiac OFT forms at the arterial pole of the heart tube, connecting the ventricles to the aortic sac. The OFT is composed of an outer myocardial layer, derived from the anterior SHF, and an inner lining of endocardial cells. As the primitive heart tube undergoes rightward looping, SHF progenitors will migrate
progressively from the pharyngeal mesoderm to elongate the heart tube and contribute to the myocardial layer of the OFT, which reaches its maximal length by E10.5.\textsuperscript{2} As the heart tube grows, the AV canal, future atrial and ventricular septa and the developing OFT will align with the ventricles, facilitating proper septation of the heart. Abnormalities in this convergence process often cause major OFT defects.\textsuperscript{77} The OFT also undergoes a wedging process where it rotates in a counterclockwise direction, putting the aortic valves behind the pulmonary trunk and to the left to align between the two AV valves. Abnormalities in the wedging process leads to misalignment of the OFT with the ventricles and failure to complete development of the interventricular septum.\textsuperscript{78} As SHF progenitors add to the early OFT, proliferation markers are downregulated and early markers of the myocardial lineage are upregulated.\textsuperscript{79} The early OFT is divided into two parts: conal/proximal region, which is adjacent to the RV, and the truncal/distal region, which is adjacent to the pharyngeal arches. These two regions of the OFT have distinct characteristics and the boundary between the truncal and conal parts plays crucial roles in remodeling.\textsuperscript{53}

Cardiac neural crest cells are involved in numerous events of OFT development including remodeling of the pharyngeal aortic arches, truncal cushion development, formation of the smooth muscle cell layer and reciprocal signaling to the SHF to regulate SHF proliferation and deployment.\textsuperscript{36, 80} As the truncal portion of the OFT forms, cardiac neural crest cells will delaminate from the neural tube at approximately E9.0, migrate into the caudal pharyngeal arches and colonize the truncal OFT cushions. These cardiac neural crest cells, together with fusion of the truncal cushions of the OFT, will give rise to the aorticopulmonary septation complex by E11.0.\textsuperscript{81} The septation complex will form a
shelf-like structure distal to the OFT, between the fourth and sixth aortic arches, growing towards the proximal OFT and fusing with the conal OFT cushions to septate the common OFT. As the same time, the OFT undergoes counterclockwise rotation and shifts to become aligned above the future ventricular septum. The conal cushions also fuse to divide the proximal OFT into right and left ventricular outlets. The proximal outlet septum becomes muscularized through myocardialization, a process where myocardial cells grow and migrate into the flanking mesenchymal tissue. By E14.5, OFT remodeling and septation is complete and the aorta is connected to the LV and pulmonary artery is connected to the RV.

1.1.6 Transcription Factors in Heart Development

Transcriptional activity and gene expression during heart development is a tightly controlled process, involving cascades of interacting transcription factors. A transcription factor has sequence-dependent affinity for specific DNA motifs to regulate transcriptional activity of numerous downstream target genes. A core group of transcription factors can interact with each other or function as biochemical partners in a complex transcriptional network to regulate each step of heart development. Some key cardiac transcription factors and the most well studied to date include TBX5, NKX2.5 and GATA4. Based on the multifaceted transcriptional interactions and synergistic regulation of numerous pathways by transcription factors, it is not surprising that a mutation in any one of the core transcription factors has been shown to cause inherited or sporadic congenital heart defects (CHDs) in humans.

One of the first transcription factor genes identified to be associated with inherited CHDs was TBX5. Humans with a heterozygous mutation in TBX5 have Holt-Oram
syndrome, which include defects in upper limb and heart development, specifically atrial septal defects (ASDs), ventricular septal defects (VSDs) and conduction defects.\textsuperscript{88, 89} In mouse studies, TBX5 has been shown to regulate interventricular septum formation and development of the conduction system.\textsuperscript{90, 91} Mice with a \textit{TBX5} null mutation have developmental arrest by E9.5, the heart tube fails to loop and the embryos do not survive past E10.5. It was shown that TBX5 and NKX2.5 interact to regulate synergistic transcriptional activation of atrial natriuretic peptide (\textit{ANP}) and connexin-40 (\textit{Cx40}) genes.\textsuperscript{92} Along with that, TBX5 and NKX2.5 interact in a transcriptional cascade to regulate development and maintenance of the cardiac conduction system.\textsuperscript{93}

Mutations in the \textit{NKX2.5} gene were the first single-gene defect linked to non-syndromic CHDs in humans. Mutations in the \textit{NKX2.5} gene have been shown to cause inherited ASDs, atrioventricular block, VSDs, Ebstein’s anomaly and Tetralogy of Fallot (TOF).\textsuperscript{94, 95} NKX2.5 deficient mice are embryonic lethal by E9.0 due to defects in looping morphogenesis of the heart tube.\textsuperscript{96} Although commitment to the cardiac lineage is not compromised in NKX2.5 deficient mice, expression of several cardiac genes is reduced including \textit{ANP}, myosin light chain 2v (\textit{MLC2v}) and \textit{Hand1}, indicating that NKX2.5 functions near the top of the transcriptional cascade to regulate numerous cardiac genes essential for myocardium development beyond the looping stage.\textsuperscript{96-98} In addition to interactions with TBX5,\textsuperscript{92} NKX2.5 has also been shown to interact directly with GATA4 and another transcription factor, serum response factor (SRF), to promote cardiac sarcomeric protein expression.\textsuperscript{99}

The zinc finger transcription factor GATA4 is one of the earliest transcription factors expressed in the developing mouse heart beginning at E7.5 and continues to be
expressed throughout adulthood where it maintains normal cardiac function and acts to protect the heart from stress-induced heart failure.\textsuperscript{100-103} Studies in mice where GATA4 expression levels were reduced in varying degrees showed that GATA4 is a dosage-sensitive regulator of heart development. A graded reduction in GATA4 expression resulted in mice with a common AVC, double outlet right ventricle (DORV) and hypoplasia of the myocardium.\textsuperscript{104} Mutations in the \textit{GATA4} gene cause inherited ASD and VSDs in humans, as well as dilated cardiomyopathy in later life.\textsuperscript{105} Like the other core transcription factors, GATA4 acts in a synergistic manner with other transcription factors, such as Smad4 and Mef2 proteins, to regulate valve development and expression of cardiac genes.\textsuperscript{106, 107} In addition, GATA4 functionally interacts with TBX5 to activate expression of \textit{Cx30.2}, a gap junction protein highly expressed in the AV node.\textsuperscript{108}

In addition to these core transcription factors, TBX5, NKX2.5 and GATA4, other notable transcription factors that are discussed in this thesis include the basic helix-loop-helix (bHLH) transcription factor Hand2 and TBX20. Hand2 is highly expressed in the developing RV and \textit{Hand2}\textsuperscript{+/−} mice are embryonic lethal by E10.5, displaying RV hypoplasia and vascular defects.\textsuperscript{109-111} Along with its previously mentioned interactions, NKX2.5 also interacts with Hand2 and Mef2c to regulate ventricular identity.\textsuperscript{112, 113} The Hand factors, Hand1 and Hand2, function in a dose-sensitive manner to regulate cardiac development and gene expression.\textsuperscript{114} \textit{Hand2} maps to chromosome 4q33, where duplications or mutations have been associated with a range of septal and OFT defects.\textsuperscript{115} In a cohort of patients with defects in the RV and OFT, 5\% had mutations in the \textit{Hand2} gene, making it a strong candidate gene related to CHDs.\textsuperscript{116} TBX20 is expressed in both the FHF and SHF and acts as a dose-sensitive regulator of OFT, valve and RV
TBX20 acts to repress TBX2 in the outer curvature to establish regional patterning of the myocardium during development and functions to maintain adult heart function. $TBX20^{-/-}$ mice fail to undergo heart tube looping and chamber formation does not occur.\textsuperscript{119,120}

Research in the past two decades has rapidly uncovered an intricate cardiac transcriptional network involving numerous transcription factors that is beyond the scope of this thesis. Although the importance of these transcription factors in heart development have been established, few downstream targets have been identified that can explain the precise cellular mechanisms for CHDs. Further research to identify the specific targets downstream of the associated transcription factors will be crucial for understanding normal heart development and the pathogenesis of CHDs.\textsuperscript{121}

### 1.2 Congenital Heart Defects

Congenital heart defects (CHDs) are abnormalities in the structure and function of the heart that arise during embryonic development. It is the most common human birth defect, affecting up to 5\% of live births and it is the leading cause of death from a congenital malformation in the first year of life in industrialized nations.\textsuperscript{122,123} Tremendous advances in diagnostics, surgical techniques and medical care have increased the survival rate of CHD patients in the last 60 years. Due to these improvements in care, the population of adult CHD patients has grown rapidly and is now estimated to outnumber the childhood population 66\% to 34\%, respectively, in Canada.\textsuperscript{124} However, adult patients can face ongoing health and disease burdens throughout their lives including multiple re-operations, arrhythmias, bacterial endocarditis and heart failure, which are major causes of morbidity and mortality.\textsuperscript{125} Our
current understanding of the etiology of CHDs is still limited as only 20% of cases can be attributed to a known cause. CHDs can be linked to genetic or environmental factors, or a combination of both. Some environmental factors include maternal alcohol consumption, use of therapeutic drugs, and maternal illnesses and conditions such as diabetes and rubella infection. Genetic factors include chromosomal disorders like trisomy 21, deletion syndromes such as DiGeorge Syndrome or single gene disorders like Holt-Oram caused by $TBX5$ mutations. Despite much progress in our understanding of heart development, the cellular and molecular mechanisms underlying CHDs are still not fully understood. A further understanding of the intricate developmental mechanisms regulating cardiogenesis is critical for potential future advancements in diagnostics, prevention and treatment of CHDs.

1.2.1 Atrial and Ventricular Septal Defects

Holes that exist between the atria or ventricles and allow mixing of oxygenated and deoxygenated blood between the two chambers are defined as septal defects. When accounting for all the different types of CHDs, defects in ventricular and atrial septation are one of the most common types, with reported prevalence of 30.7% for VSDs and 11.6% for ASDs. A VSD can occur in either the muscular or membranous part of the ventricular septum, with membranous VSDs occurring more frequently. VSDs can also occur as an isolated cardiac defect or as part of a complex CHD, such as TOF and transposition of the great arteries. ASDs can be divided into two major types: ostium primum or ostium secundum defect. If the primary atrial septum or the secondary atrial septum fails to form properly, an ostium secundum defect occurs. If the ostium secundum defect is large, it may lead to right atrial overload due to left-right shunting and cause
complications such as atrial fibrillation and pulmonary hypertension.\textsuperscript{130} Ostium primum defect is less common but more severe compared to ostium secundum defects. This defect is an incomplete septation between the inferior margin of the primary atrial septum and arises from abnormal growth of the primary septum or development of the endocardial cushions.\textsuperscript{131} Like other CHDs, the etiology of atrial and ventricular septal defects is complex and multifactorial. As described above, mutations in cardiac transcription factors TBX5, NKX2.5 and GATA4 are associated with VSDs and ASDs in humans. Functional interaction of these cardiac transcription factors is critical for septal development.\textsuperscript{88, 94, 105, 132} Besides a genetic factor, environment influences such as pregestational diabetes and teratogens are associated with septation defects in the fetus.\textsuperscript{127} Moreover, numerous mouse studies have shown perturbations in SHF progenitor signaling pathways can lead to ASDs and VSDs, since the SHF is a major contributor of these cardiac structures.\textsuperscript{61-63}

1.2.2 Ventricular Noncompaction Defects

Aberrant ventricular chamber maturation can lead to left ventricular non-compaction (LVNC), which is characterized by excessive trabeculae, deep recesses within the ventricular cavity and a thin compact myocardium. This defect is called LVNC because it affects mostly the LV but in rare cases, this defect can also occur as an isolated incident in the RV. LVNC can occur as an isolated event or combined with other CHDs, leading to heart failure, thromboembolic events, arrhythmias and/ or sudden cardiac death.\textsuperscript{133-135} LVNC is the third most common cardiomyopathy after dilated cardiomyopathy and hypertrophic cardiomyopathy.\textsuperscript{136} Numerous studies have shown that ventricular noncompaction arises from impaired maturation or arrest of the developing
ventricular myocardium. This defect has been linked to mutations in mitochondrial proteins, along with cytoskeleton, Z-line and sarcomeric proteins. For example, in one family with LVNC, a mutation in the cardiac troponin T gene was found. However, recapitulation of this mutation in a mouse model did not cause LVNC. Overall, this type of defect is still relatively not well understood and there seems to be a lack of a genotype-phenotype link, suggesting genetic heterogeneity in patients.\textsuperscript{137, 138} Numerous mouse models have been developed in the past 10 years to study the genetic and molecular mechanisms of LVNC including the Noonan syndrome SHP2 gain-of-function mutation mouse and mouse with myocardial deletion of MIB1, a gene involved in the Notch pathway.\textsuperscript{139, 140} However, the defects in the ventricular myocardium are not homogenous between the mouse models, likely reflecting the complexity underlying chamber development and maturation.

1.2.3 Outflow Tract Defects

Proper development of the cardiac OFT requires complex temporal and spatial coordination between different cardiac progenitor populations, as previously described above. Due to the developmental intricacies of this part of the heart, the OFT is particularly susceptible to developmental defects. Abnormalities in OFT development account for 20 – 30% of the CHDs seen at birth in humans.\textsuperscript{141} Even after postnatal surgical correction, patients can still face health risks throughout their lives.\textsuperscript{142} The OFT defects discussed in this thesis include OFT alignment defects, which include DORV and overriding aorta, transposition of the great arteries (TGA), persistent truncus arteriosus (PTA), aortic and pulmonary artery stenosis and vascular rings.

- **Double Outlet Right Ventricle (DORV)**
During normal cardiac development, the heart tube undergoes rightward looping, convergence of the inflow and outflow poles of the heart tube and wedging of the aorta between the two atrioventricular valves. Correct alignment of the aorta and pulmonary artery with the LV and RV, respectively, is dependent on proper lengthening of the venous (inflow) and arterial (outflow) poles of the primary heart tube. Consequently, a shortened heart tube alters the alignment of the arterial pole, causing DORV, where both the pulmonary artery and aorta connect to the RV, or overriding aorta, where the aorta is positioned above a VSD (Figure 1.2B). DORV account for 1-3% of CHDs and requires surgical correction. The causes of DORV are complex and multifactorial including chromosomal and non-chromosomal genetic abnormalities and teratogenic exposures in utero. From animal studies, DORVs have been shown to arise from impaired neural crest cell and SHF progenitor contribution to the primary heart tube, leading to a shortened heart tube and subsequent defects in cardiac looping. In addition, defects in the myocardialization process have been shown to also lead to DORV. Myocardialization is a process where myocardial cells migrate and grow into the adjacent mesenchymal tissue. At E12, myocardial cells of the OFT will invade the neighbouring cushion mesenchyme, leading to a gradual replacement of the mesenchymal tissue with myocardium to form the muscular outlet septum. Failure of myocardialization leads to misalignment of the OFT and the aorta remains in contact with the RV.

- **Transposition of the Great Arteries (TGA)**

  TGA occurs when the aorta is connected incorrectly to the RV and the pulmonary artery is connected incorrectly to the LV (Figure 1.2C). TGA arises from abnormal septation of the aorticopulmonary septum between the aorta and pulmonary artery.
During normal development, the aorticopulmonary septum is formed in a spiral shape, causing the OFT to rotate and the aorta and pulmonary artery to align with their respective ventricles. TGA arises when the aorticopulmonary septum does not spiral and forms a straight, linear septum instead.\cite{78,147} TGA is estimated to occur in 3.5/10,000 live births, making it one of the more common major CHDs requiring corrective surgery shortly after birth.\cite{148} Post-surgery, later health risks into adulthood can include atrial arrhythmias, tricuspid regurgitation, RV dysfunction and pulmonary hypertension.\cite{125} The detailed pathogenesis of TGA is still relatively unknown. A study in chick demonstrated that neural crest ablation may lead to TGA but the mechanisms are unclear.\cite{149} TGA is strongly associated with heterotaxy in humans and some patients with TGA have mutations in \textit{ZIC3}, \textit{CFC1} and \textit{Nodal}, which are genes associated with laterality.\cite{150,151} Along with genetic factors, environmental factors such as maternal diabetes and infections have also been linked to development of TGA.\cite{148}

- **Persistent Truncus Arteriosus (PTA)**

PTA, also known as common arterial trunk, occurs when the OFT fails to divide into aorta and pulmonary artery during development, resulting in one common artery that arises from the ventricles (Figure 1.2D). The process of OFT septation is dependent on cardiac neural crest cells, which migrate into the distal OFT and condense to form the aorticopulmonary septation complex, dividing the common artery into two equal sized vessels.\cite{41,68} Studies have shown that ablation of cardiac neural crests in chick embryos result in PTA.\cite{152} In mouse studies, alterations to the cardiac neural crest cells also lead to PTA.\cite{153} Sema3c, a secreted axonal guidance molecule, is critical for migration and targeting of cardiac neural crest cells to the OFT. Loss of \textit{Sema3c} leads to PTA and aortic
arch defects. Other genetic mutations in mice that have been associated with PTA include the transcription factors Pax3, AP2α and Pitx2, and signaling proteins, BmprII and Alk2. PTA occurs in approximately 1% of infants born with CHDs and up to 50% of PTA cases are associated with chromosome 22q11.2 deletion syndrome. PTA is often considered the most severe type of OFT defect, as it is usually associated with a poor prognosis and complete repair is not possible in some patient cases. However, in the last 30 years, surgical procedures for PTA have improved with a 95% survival rate. Many patients often require later interventions related to RV flow obstruction and repair of the truncal valves.

• **Aortic & Pulmonary Artery Stenosis**

Stenosis of the pulmonary artery occurs when there is abnormal constriction or narrowing of the pulmonary artery vessel. This leads to development of higher pressure in the RV in an effort to overcome the narrowing of the pulmonary artery. Pulmonary artery stenosis makes up one of the four characteristics of TOF, which also include VSD, RV hypertrophy and overriding aorta. In addition, pulmonary artery stenosis is often associated with Williams syndrome, which arises from deletion of a portion of chromosome 7 and presents as connective tissue abnormalities, endocrine abnormalities and mental retardation. Treatments for pulmonary artery stenosis include balloon dilation, stent placement or surgical repair. Similar to pulmonary artery stenosis, stenosis of the aorta is a narrowing of the blood vessel. This condition is also called supravalvular aortic stenosis because the narrowing occurs above the aortic valve. This defect can be caused by mutations in the ELN gene, which is responsible for production of tropoelastin, the precursor of elastin. However, over 50% of supravalvular aortic stenosis has no known
Surgical intervention is the primary treatment for supravalvular aortic stenosis.\textsuperscript{162}

- **Vascular Ring**

A vascular ring is a vascular encirclement of the esophagus and trachea due to a failure of the aortic arch arteries to remodel during cardiac development. Vascular rings are found in less than 1% of CHDs. Some cases of vascular rings are asymptomatic while others can present with major symptoms such as compression or obstruction of the esophagus or airway.\textsuperscript{163} Up to 50% of patients with vascular rings also have other CHDs such as VSD, TOF or patent ductus arteriosus (PDA). Non-cardiovascular associations include genetic syndromes such as DiGeorge syndrome, Down syndrome and cleft lip-palate.\textsuperscript{164, 165} Several types of vascular rings exist with the two most common being double aortic arch and right aortic arch with left ligamentum arteriosum, comprising 85-95% of vascular ring cases.\textsuperscript{164, 166} During early embryonic development, the dorsal and ventral aortic arch are connected by six bilateral embryonic arches. Each arch will develop into vasculature or regress. The first, second, fifth arches and part of the fourth arch will regress. The third arch will become the common carotid arteries and the sixth arch will form the proximal pulmonary artery and ductus arteriosus. Vascular rings can arise when development or regression of one or more of the arches does not occur normally.\textsuperscript{167} During development, a subpopulation of cardiac neural crest cells will migrate into pharyngeal arches three, four and six to remodel the arteries. Failure of cardiac neural crest cells to populate and remodel this region can lead to vascular rings.\textsuperscript{168}
Figure 1.2. Schematic representation of outflow tract defects.

In the normally developed heart, the OFT is fully septated. The aorta (Ao) connects to the LV and the pulmonary artery (PA) connects to the RV (A). In cases of DORV, both the aorta and pulmonary artery connect to the RV (B). In TGA, the aorta and pulmonary have switched connections to their respective ventricles, where the Ao now connects to the RV and the pulmonary connects to the LV (C). When the OFT fails to septate, a PTA occurs (D). For all defects shown in B-D, there is an obligatory VSD.

1.3 Rac1 GTPase
1.3.1 Rac1 Overview

Rac GTPases are small (20-30 kDa) monomeric, signaling GTP-binding proteins, which are a subfamily of the Rho GTPase family. The Rho GTPase family is comprised of 8 subfamilies encompassing a total of 20 intracellular signaling molecules. The Rac family contains 4 members: Rac1, Rac2, Rac3 and RhoG. \(^{169, 170}\) Rac proteins play an important role in various cellular events, including actin cytoskeletal reorganization and lamellipodia formation for migration, transformation, proliferation, cell survival, gene expression, endocytosis and trafficking and superoxide production.\(^{171-175}\) Rac1, Rac2 and Rac3 have similar genetic sequences with different expression patterns. Rac1 is ubiquitously expressed, whereas Rac2 expression is restricted to cells of hematopoietic
and Rac3 is most abundant in the brain. RhoG has the lowest sequence similarity to Rac1 and is ubiquitously expressed. The most well studied and characterized protein of the Rac family is Rac1.

### 1.3.2 Activation of Rac1

Rac1 act as a molecular switch, cycling between active (Rac1-GTP) and inactive (Rac1-GDP) forms on the cell membrane. Under basal conditions, inactive Rac1 localizes in the cytosol and is bound to a GDP dissociation inhibitor (GDI). Upon stimulation by various factors such as cytokines, Rac1-GDP dissociates from GDI and attaches to the membrane. Two major classes of proteins facilitate binding and hydrolysis of GTP to GDP on Rho GTPases, which are guanine nucleotide exchange factors (GEFs) and the GTPase activating proteins (GAPs), respectively.

GEFs become activated and will open the Rac1 nucleotide-binding site to allow the exchange of GDP for GTP and subsequent activation of downstream effectors. Activation of GEFs through upstream activation signals can occur through several different mechanisms, which include protein-protein or protein-lipid interactions, binding of second messengers and posttranslational modifications. For example, a common activator of Rac-GEFs is phosphoinositide 3-kinase (PI3K) which induces GEF membrane translocation and/or conformational changes. Some Rac-GEFs known to be activated through PI3K include P-Rex, SWAP-70, Tiam1, Pix, Sos and Vav. Some common second messengers can also activate GEFs, including cyclic adenosine monophosphate (cAMP), Ca\(^{2+}\) and diacylglycerol (DAG). Over 70 GEFs have been reported in humans and in most cases, each GEF can activate multiple Rho GTPases, creating a network of complex crosstalk between GEFs and Rho GTPase proteins.
example, the GEF Tiam1 can activate Rac1, Rac2 and Rac3 while another GEF, Vav1, can also activate Rac1 along with RhoA, RhoG and Cdc42. Similar to GEFs, over 80 GAPs regulate Rho GTPases and can act on multiple Rho GTPases. Although GTPase proteins have their own intrinsic GTP hydrolysis activity, the reaction speed is slow and interaction with a GAP can accelerate the GTP hydrolysis reaction by several orders of magnitude (Figure 1.3). To account for the large numbers of GEFs and GAPs compared to Rho GTPase proteins, it has been shown that some GEFs and GAPs have spatial-temporal specific expression and may regulate a specific Rho GTPase signaling pathway.

Figure 1.3. Schematic representation of the Rac1 activation cycle.

Rac1 will cycle from an inactive GDI-bound state in the cytosol to an inactive GDP-bound state on the cell membrane. GEFs enable the exchange for GDP for GTP to
activate Rac1, which will then activate downstream effectors. GAPs facilitate the exchange of GTP for GDP, thus inactivating Rac1.

1.3.3 Pleiotropic Effects of Rac1

Activated Rac1 interacts with a multitude of effectors to activate downstream signaling cascades and influence numerous, diverse physiological responses. Some effectors of activated Rac1 include p67phox, IQ motif containing GTPase activating proteins (IQGAPs), p21 activating kinases (PAK), Wiskott-Aldrich syndrome protein with a V-domain (WAVE), and partitioning-defect-6 (PAR-6).\textsuperscript{171, 189} Interaction of activated Rac1 with p67phox leads to activation of NADPH oxidase and production of superoxide.\textsuperscript{190} IQGAPs stabilize active Rac1 and acts as a scaffolding protein to regulate cell adhesion, actin cytoskeleton and cell cycle progression signaling pathways.\textsuperscript{191} Cell adhesion and cytoskeleton dynamics can also be partially regulated through PAKs, along with transcription and cell survival.\textsuperscript{192} Binding of activated Rac1 to WAVE protein leads to activation of the actin-related-protein-2/3 (Arp2/3) complex which promotes actin polymerization from the sides of existing filaments, creating a branched-actin-filament network and lamellipodia formation.\textsuperscript{170} Activated Rac1 can also interact with PAR-6, which forms a complex with PAR-3 and the Ser/Thr kinase atypical protein kinase C isoform PKC (aPKC). PAR-6, PAR-3 and aPKC form the PAR complex, which is involved in various aspects of cell polarity.\textsuperscript{193} Rac1 can also promote gene expression through several different pathways, including NF-κB and PAK.\textsuperscript{194, 195} Through PAK, Rac1 can mediate c-Jun N-terminal kinase (JNK) regulated canonical Wnt-signaling, leading to downstream TCF/β-catenin transcriptional signaling.\textsuperscript{196} As well, studies have shown that Rac1 activation of NF-κB, JNK and p38 mitogen-activated protein kinase
(MAPK) can induce expression of the transcription factor activator protein-1 (AP1), leading to expression of genes that regulate cell cycle progression. Hence, Rac1 is a critical component of a complex signaling network in normal physiology and abnormal Rac1 signaling has been implicated in various pathophysiologies including cardiac hypertrophy, heart failure and atherosclerosis (Figure 1.4).

![Diagram of Rac1 signaling pathway]

**Figure 1.4. Downstream effectors of Rac1.**
Activated Rac1 signals to numerous downstream effectors to regulate diverse cellular functions.

### 1.3.4 Rac1 and Embryonic Development

*Rac1−/−* mice are embryonic lethal by E9.5 with defects in germ layer formation due to reduced cell adhesion and motility and apoptosis of the mesoderm tissue. Due to the early lethality of *Rac1−/−* mice, many tissue specific *Rac1* conditional knockout mouse models using the Cre/loxP system have been generated to study tissue specific roles of
Rac1. To date, Rac1 has been shown to be involved in numerous cellular processes in the development of many tissues and organs. Mice with an endothelial-specific Rac1 excision are embryonic lethal by E9.5 with development defects of the major vessels and a complete absence of small branched vessels. Endothelial cell-specific deletion of Rac1 blocks cell migration, adhesion, lamellipodia formation, permeability and tubulogenesis. In addition, mice with a forebrain-specific loss of Rac1 exhibit decreased proliferation and migration of cortical neurons during development and increased apoptosis of progenitors. During embryogenesis, specification of the anterior-posterior body axis depends on migration of an extra-embryonic organizer known as the anterior visceral endoderm (AVE). Rac1 is critical for specification of the anterior-posterior axis by acting cell-autonomously within the migrating AVE cells to extend projections and change cell shape. Rac1−/− mice fail to specify an anterior-posterior axis since AVE cells do not migrate. In the mouse epiblast, deletion of Rac1 results in embryonic lethality by E8.5. These mice have decreased migration of the mesoderm tissue and large scale cell death leading to defects in embryonic morphogenesis including failure of the lateral heart anlage to fuse, defects in closure of the neural tube and defective paraxial mesoderm formation. Regarding cardiac development, when Rac1 is specifically deleted in neural crest cells, OFT cushions are abnormally positioned, fused and smaller. In these neural crest cell Rac1 mutants, the pharyngeal arches also failed to remodel and the OFT remains unseptated, resulting in a common arterial trunk. Post-migratory Rac1-deficient neural crest cells also have an increased level of apoptosis and decreased proliferation, along with defective cell organization. To date, the role of Rac1 in other cardiac progenitor cells or cardiac tissues during development is unclear.
One study by Wei et al., used a transgenic mouse line overexpressing RhoGDIα under control of the cardiac-specific α-myosin heavy chain (αMHC) promoter. RhoGDIα is an endogenous inhibitor of many Rho protein members including RhoA, RhoB, Rac1, Rac2 and Cdc42. RhoGDIα transgenic mice were found to have defects in cardiac looping and ventricular maturation along with reduced cardiomyocyte proliferation. This study established Rho proteins as critical regulators of cardiac development; however, the roles and involvement of each specific Rho protein remained unclear. Therefore, the overall three aims of this project were to determine the specific roles of Rac1 during embryonic heart development.

1.4 Cell Polarity

Cell polarity is the asymmetrical organization of cell membrane proteins, intracellular organelles and the actin cytoskeleton to influence cell shape and structure and to regulate specialized cellular functions. Almost every eukaryotic cell possesses polarity, which is coordinated spatially and temporally and regulated by numerous signaling cascades and cytoskeleton dynamics. Many biological processes including proliferation, apoptosis, cell shape and morphology, migration and differentiation are coordinated and linked to polarization of a cell. In addition to polarity within an individual cell, polarity between neighbouring cells must also be coordinated in order to form functional tissues and organs. Through cell polarization, diverse cell types with specific functions are formed within an organism. A prime example is neurons, which have a dendritic end for synaptic inputs and an axonal end for signaling outputs. Many neurons will connect with each other to form a complex network to propagate signals throughout the body. Cell polarity is established through conserved polarity proteins
complexes, which include partitioning defect (PAR), Crumbs and Scribble. These polarity protein complexes will respond to extrinsic and intrinsic cues such as growth factor gradients and cytoskeleton dynamics, respectively, to establish cell polarity. Interestingly, recent studies have shown a critical requirement for crosstalk between polarity protein complexes and effectors of small Rho GTPases in regulation of polarity in several cell types including neurons, epithelial cells and T cells.\textsuperscript{193} For example, tight junction formation in keratinocytes is dependent on the activation of PAR-3, a component of the PAR complex, by the Rac1-GEF, Tiam1.\textsuperscript{209} Numerous studies have shown a direct link between aberrant cell polarization and pathophysiologies such as developmental defects in neural tube closure and cancer.\textsuperscript{210-212}

1.4.1 Types of Cell Polarity

Different types of cell polarity exist including front-rear polarity seen in polarized migrating cells, apico-basal polarity in epithelial cells and planar cell polarity (PCP). A cell can take on different polarity characteristics depending on developmental cues and spatial and temporal factors. For example, in formation of epithelial cells during development, migratory mesenchymal cells undergo a mesenchymal-to-epithelial transition (MET), transitioning from a migratory cell with front-rear polarity to one of an epithelial cell with apical-basal polarity. This polarity transition involves dynamic changes to the actin cytoskeleton, protein localization and trafficking pathways.\textsuperscript{213}

• **Front-Rear Polarity**

Front-rear polarity exists when the front leading edge and the back end of a migrating cell show morphological and functional asymmetry.\textsuperscript{206, 214} The Rho GTPases, Cdc42,
RhoA and Rac1, are distinctly localized to the front (Rac1 and Cdc42) or back (RhoA) while centrosomes, microtubules and Golgi are oriented towards the front of a migrating cell. Polarity protein complexes also tend to be localized to the leading edge.\textsuperscript{215, 216} Cellular functions such as protein trafficking are also polarized in a migratory cell. Through the endocytic pathway, integrins are redistributed from the back to the front of a migrating cell. The exocyst complex, which tethers transport vesicles to sites of fusion, is localized to the front of a migratory cell and is responsible for exocytosis and endocytosis of integrins in the front.\textsuperscript{217, 218} This distinct organization between the front leading edge and back of cells is intrinsic to migratory ability (Figure 1.5A).\textsuperscript{214}

- **Apical-Basal Polarity**

Apical-basal polarity is cellular asymmetry along the apical-basal cell axis and is a major feature of epithelial cells. Mammals have over 150 different types of epithelial cells that play critical roles in embryo morphogenesis, along with formation and function of numerous organs.\textsuperscript{219} The apical membrane domains of epithelial cells face the lumen or external environment while the basolateral membrane domain maintains contact with neighbouring cells or the underlying ECM. The correct localization of polarity proteins and cell-cell junctions is crucial for establishment of polarity and for designating the apical and basolateral domains. The PAR and Crumbs polarity protein complex define the apical domain while the Scribble polarity protein complex is found in the basolateral domain.\textsuperscript{220, 221} Cell-cell junctions, which include adherens junctions, tight junctions, gap junctions and desmosomes, are located at distinct regions along the apical-basal axis. Tight junctions prevent the diffusion of proteins between the apical and basolateral surfaces, thus maintaining apical-basal polarity. Adherens junctions, found basal to tight
junctons, form a band that encircles the cell and provide adhesion between neighbouring epithelial cells. The formation of both adherens junctions and tight junctions are highly interconnected where formation of early adherens junctions are a prerequisite for tight junction formation.\textsuperscript{222, 223} Along with structural polarity, functional polarity involving ion channels also exist to create a sodium gradient to move other ions and solutes across the epithelium. A polarized epithelial layer permits functional compartmentalization, allowing exchange of nutrients and waste between the internal and external environments (Figure 1.5B). A wide range of signaling pathways have been implicated in regulation of apical-basal polarity including TGFβ, integrin, Hippo and Wnt.\textsuperscript{224, 225} Establishment of apical-basal polarity is also linked to numerous cellular processes including proliferation, cell morphology and maintenance of overall tissue architecture.\textsuperscript{226} Loss of apical-basal polarity in epithelial cells is the source of approximately 90\% of all human cancers.\textsuperscript{227}

- **Planar Cell Polarity**

PCP is the alignment of cells across a tissue plane. PCP is orthogonal to the axis of apical-basal polarity and occurs between adjacent cells, allowing for coordinated alignment of numerous cells in a tissue. The PCP signaling pathway is well understood in *Drosophila* and many of the signaling components are well conserved in vertebrates and mammals. Various processes such as gastrulation, orientation of stereocilia within the inner ear, neural tube closure and oriented cell division are dependent on PCP genes.\textsuperscript{228-231} PCP can be divided into two distinct molecular systems: a global group of proteins (Fat, Dachsous and Four-jointed), which regulate polarity in the direction of the body axis and a core group of proteins (Frizzled, Dishevelled, Prickle, Van Gogh, Diego and Flamingo), which regulate polarization signals within individual cells. A common feature
of both the global and core system is the asymmetrical distribution of their components (Figure 1.5C).

The PCP pathway will signal to downstream effectors that include RhoA and Rac1, leading to changes in the actin cytoskeleton and generation of morphological asymmetry (Figure 1.6). In vertebrates, non-canonical Wnts, which include Wnt5a and Wnt11, will bind to the Frizzled receptor. This leads to recruitment of Dishevelled to the plasma membrane and activation of diverse pathways. Specifically, Dishevelled binds Daam1 (DVL-associated activator of morphogenesis 1) and activates RhoA to regulate the actin cytoskeleton in cell polarity and migration. In a parallel PCP pathway, Dishevelled binds to Rac1 to signal through JNK, leading to gene transcription and changes in the actin cytoskeleton and cell polarity. Disruptions to PCP signaling can cause severe developmental defects including skeletal defects, left/right patterning defects and ciliopathies.
Figure 1.5. Different types of cell polarity.
(A) Rho GTPases, polarity protein complexes, along with other cellular components have a polarized distribution, creating front-rear polarity in a migratory cell. (B) Epithelial cells have apical-basal polarity. The apical and basal domains have distinct localization of polarity protein complexes, cell-cell junctions and ion channels to regulate epithelial function. (C) PCP is the alignment of adjacent cells within the plane of a cell sheet. Core PCP proteins have an asymmetrical distribution within a cell, which is critical for establishment of PCP in a tissue. C, centrosome; TJ, tight junction; AJ, adherens junction.

Figure adapted with modifications from Nelson, WJ. *Cold Spring Harb Perspect Biol.* 2009;1:a000513 and Devenport, D. *J Cell Biol.* 2014;207(2):171-179.

**Figure 1.6.** The outcome of PCP: Actin cytoskeleton organization, cell shape, migration and alignment.
Core PCP proteins in the posterior end of a cell (Frizzled, Diego and Dishevelled) signal to downstream effectors, which include Rac1 and RhoA, leading to actin reorganization and overall polarization of cells.

1.4.2 Rac1 & Cell Polarity

Rac1 signaling has been shown to be a critical regulator of several cell polarization events including polarized migration, apical-basal polarity and PCP. The crucial role of Rac1 in establishment of cell polarity is not surprising, given its established function in actin polymerization and regulation of cytoskeleton dynamics.170

In front-rear polarity, proper polarization and directed cell migration requires Rho GTPase activity and crosstalk with polarity proteins. Cell migration is the net outcome of Cdc42 and Rac1-directed formation of cell protrusions at the front leading edge and RhoA-directed retraction of adhesion complexes at the rear of a migrating cell.238 In migratory cells, activated Rac1 will localize at the front leading edge to form adhesions and activate WAVE and Arp2/3 protein complexes, leading to subsequent actin polymerization and lamellipodia formation. RhoA is mutually antagonistic to the activity of Rac1 and is most active at the rear and sides of a cell, responsible for actomyosin contraction necessary for cell detachment during migration.215, 216 In addition, the polarity protein complexes, PAR, Crumbs and Scribble, are located at the leading edge of a migrating cell.215, 216 Scribble can localize Rac1 to the front edge, promoting subsequent polarization of the Golgi apparatus and cell migration.239 Decreased Scribble expression can lead to reduced Rac1 activation, indicating a direct or indirection regulation of Rac1 by Scribble.211 Defects in front-rear polarity induced through inhibition of the small
GTPases, Rac1 or RhoA, or disruptions in cytoskeleton organization have been shown to result in global loss of migratory ability in neutrophils.\textsuperscript{214}

Spatiotemporal regulation of Rac1 signaling has been demonstrated to be important for correct apical-basal polarization. Rac1 activity is critical for directing where the apical domain will form. Rac1 promotes assembly of the laminin basement membrane by regulating dystroglycan or β1-integrins, thus generating the correct tissue architecture to orient apical-basal polarity. Blocking Rac1 activity and laminin assembly leads to inversion of apical-basal polarity.\textsuperscript{240, 241} Activity of Rac1 is also differentially regulated along the apical-basal axis, with activity being higher at adherens junctions and lower at tight junctions, which is crucial for cell-cell junction formation.\textsuperscript{242, 243} Clustering of E-cadherin at sites of cell-cell contact formation leads to activation of Rac1. Local Rac1 activity will drive the formation of actin-based protrusions, leading to formation of lamellipodia and promotion of E-cadherin-mediated adherens junction formation with neighbouring cells.\textsuperscript{244, 245} In addition, activation of the PAR polarity complex by Rac1-Tiam is required for tight junction formation.\textsuperscript{246} Aberrant Rac1 activity can disrupt adherens junction formation and perturb tight junction function, causing overall loss of apical-basal polarity. Thus, proper formation of cell junctions and establishment of apical-basal polarity requires tight regulation of Rac1 activity, coinciding with correct E-cadherin localization and regulators of the actin cytoskeleton.\textsuperscript{247, 248}

Overall tissue organization and polarity is also regulated in part by Rac1 signaling. However, the downstream effectors of PCP can vary between organisms and tissues. In \textit{Drosophila} eye development, Frizzled and Dishevelled will signal to Rac1, leading to nuclear activation through JNK.\textsuperscript{249} In vertebrates, non-canonical Wnts (Wnt5a
and Wnt11) will bind to the transmembrane receptor, Frizzled, leading to recruitment of the scaffolding protein, Dishevelled and downstream activation of Rac1. Rac1 has been shown to be a crucial effector of the PCP pathway during convergent-extension movements in gastrulation. In addition, the core transmembrane PCP protein, Vangl2, recruits Rac1 to regulate adherens junctions, which is critical to neural tube closure. Rac1 signaling through PAK and downstream regulation of actin dynamics play a critical role in morphogenesis of the auditory sensory epithelium and stereociliary bundle of the inner ear. Conditional loss of Rac1 activity in the developing inner ear lead to overall misorientation and misalignment of hair bundles, similar to what is observed in PCP mutants.

1.4.3 Cell Polarity & Heart Development

Development of the heart is a dynamic process involving changes in cell shape and morphology, cell orientation and migration and tissue remodeling. Cell polarity is implicated early on in cardiac development during migration of cardiac progenitors to the embryonic midline and establishment of the early heart tube. In early development, cardiomyocytes are a spherical, cuboidal-like shape and junctional proteins are distributed uniformly throughout the cell membrane. By E13.5, cardiomyocytes start to elongate and cell junctional proteins will begin to rearrange within the membrane. Following two weeks after birth, cardiomyocytes will continue the polarization process to acquire the typical rod-shape of adult cardiomyocytes, undergoing progressive alignment with neighbouring cardiomyocytes. Junctional proteins, which include adherens junctions and desmosomes that anchor actin filaments and intermediate filaments, respectively, will become restricted to the intercalated discs by P10. Gap junction proteins have a distinct
pattern from junctional proteins and are only restricted to the intercalated discs by one month after birth. Beyond the cellular level, a recent study showed that within the developing embryonic myocardium, cardiac cells are locally coordinated and preferentially align and divide in a plane parallel to the outer surface of the heart. In addition, different areas of the ventricle have various orientations of cardiomyocyte growth, following a circumferential, radial or perpendicular orientation relative to the ventricular wall. Cardiomyocytes in the subendocardial and subepicardial layers form helical sheets while cardiomyocytes in the middle layer form a circular sheet. Although the cardiomyocyte polarization process has been well documented, the signals regulating and coordinating this process of polarization during embryonic and postnatal development are still relatively unclear.

Recent studies have demonstrated a critical role for PCP in embryonic heart development. Specifically, mutations in Vanl2 or Scribble, components of the PCP pathway, led to abnormal organization and noncompaction of the ventricular myocardium. The developing cardiomyocytes failed to elongate and polarize, instead, retaining a spherical shape in PCP mutants. As well, common to many PCP mutants was a defect in septation and alignment of the great arteries with their respective ventricles, strongly supporting a critical role for PCP signaling in OFT development. However, these mutation studies were unable to determine the major cell types in which PCP signaling was required for cardiac development. Subsequent studies involving tissue-specific deletions of the PCP genes, Dvl1/2, Vanl2 and Wnt5a revealed a critical requirement for PCP signaling in SHF cells. Disruption of PCP signaling in the SHF resulted in defects in SHF progenitor cell deployment, shortened OFT length, defects in
cardiac looping and abnormal SHF progenitor cell morphology and behaviour.\textsuperscript{266-268} Interestingly, the importance of PCP signaling during cardiac development seems to be species and cell type dependent. Although a critical requirement for PCP signaling in neural crest cells was described in fish and frogs, the role of PCP signaling in mouse neural crest cells is still relatively unclear.\textsuperscript{269, 270} \textit{Vangl2} (\textit{looptail}; \textit{Lp/Lp}) mutant mice lack PCP signaling and fail to complete neural tube closure. However, neural crest cell specification and migration occurs normally in the \textit{Lp/Lp} mice.\textsuperscript{269} In addition, although \textit{Lp/Lp} mice have CHDs, migration of cardiac neural crest cells into the developing OFT was shown to be normal.\textsuperscript{263} In contrast, \textit{Dvl2} mouse mutants were reported to have OFT defects and displayed abnormalities in cardiac neural crest cell migration into the developing heart.\textsuperscript{271} However, a subsequent study using the cardiac neural crest specific \textit{Wnt1-Cre} mouse to delete \textit{Dvl1} and \textit{Dvl2} found no CHDs or defects in cardiac neural crest cells.\textsuperscript{266} Potential explanations for these conflicting findings regarding PCP signaling in mouse neural crest cells include functional redundancy between PCP genes and/or secondary defects to other cell types, such as the SHF cells. Further studies with cardiac neural crest specific deletions of PCP genes should be performed to determine the exact role of the PCP pathway in mouse neural crest cells.

In addition to PCP, SHF progenitors also exhibit characteristics of apical-basal polarity. Before discovery of the SHF, a pioneering study in 1973 was one of the first to describe a population of cells adjacent to the distal border of the heart that formed a cuboidal epithelium of mesenchymal cells.\textsuperscript{272} In later studies in chicks, SHF progenitors were described to possess a pseudo-stratified columnar layer of epithelium.\textsuperscript{273} This organized, epithelial characteristics of SHF progenitors with apical-basolateral domains,
tight junctions and adherens junctions, apical monocilia and dynamic basal filopodia has also been confirmed in subsequent mouse studies.\textsuperscript{268, 274} The transcription factor, TBX1, was shown to be a critical regulator of SHF apical-basal polarity and cell morphology. \textit{TBX1} regulates proliferation and differentiation of the SHF and is the major gene affected in DiGeorge syndrome (DGS). DGS manifests from heterozygous deletion of a region of chromosome 22q11.2 causing OFT defects along with other developmental defects.\textsuperscript{275, 276} Loss of TBX1 resulted in cell shape changes in the SHF, decreased basolateral membrane domain and impaired filopodia activity, demonstrating a critical link between the epithelial properties of SHF progenitors and OFT development.\textsuperscript{274} However, the role of Rac1 in regulation of cell polarity in SHF progenitors and OFT development is not clear.

1.5 Rationale and Hypothesis

CHDs are the most common type of congenital defect in humans, representing 28\% of all major congenital anomalies worldwide.\textsuperscript{277} Medical advancements have dramatically increased the survival rate of patients born with these types of defects. However, CHD adult patients often need long-term expert medical care and treatment, making this disease a major global health burden.\textsuperscript{278} The origins of CHDs are often complex and multifactorial, with both environmental and genetic contributions. Despite much progress in our understanding of the molecular and cellular mechanisms of heart development through the use of various animal models, our understanding of the etiology of CHDs is still incomplete.\textsuperscript{127, 128}

In recent years, research has demonstrated a critical role for cell polarity in embryonic development of the heart. Mature cardiomyocytes are oriented and organized
in a polarized manner within the heart but the signaling pathways regulating this polarity are not well known. Using mouse models, several studies showed that SHF progenitors require PCP signaling for proper development of cardiac structures such as the OFT and ventricular myocardium. In addition, SHF progenitors form an apicobasally-polarized epithelium in the dorsal pericardial wall before migration and differentiation in the heart tube. Disruption of this SHF polarity at an early stage impacts downstream development of SHF-derived cardiac structures. Rac1 is a pleiotropic effector of numerous cellular processes including actin polymerization and regulation of cytoskeleton dynamics, which are processes involved in regulation of cell polarity. The role of Rac1 has been demonstrated in various aspects of cell polarity including front-rear polarity during neutrophil cell migration, orientation of epithelial apical-basal polarity and in PCP-regulated convergent-extension movements during gastrulation. However, the role of Rac1 and regulation of cardiomyocyte polarity during heart development is unknown. Thus, the main goal of this thesis project is to understand the role of Rac1 in cell polarity during heart development. **The overall hypothesis of this thesis is that Rac1 is required for cardiomyocyte polarity and embryonic heart development.**

1.5.1 Aim 1: Rac1 and Cardiomyocyte Polarity

The first aim of this thesis was to study the role of Rac1 in cardiomyocyte polarity and embryonic heart development using a mouse line with an anterior SHF-specific Rac1 deficiency, $Rac1^{SHF}$. Early cardiomyocytes have a cuboidal shape and will gradually take on a polarized, elongated morphology by two weeks postnatally in the mouse. Junctional proteins go from being uniformly distributed in embryonic cardiomyocytes to restricted
at the ends of the rod-shaped adult cardiomyocytes. What regulates this cardiomyocyte polarization process is still not fully understood. Rac1 is involved in reorganization of the actin cytoskeleton, which drives cell shape changes and migration. Studies have shown that PCP mutants have cardiomyocytes that fail to elongate and polarize during development. However, whether Rac1 is a downstream effector of the polarity pathways that exist in developing cardiomyocytes is unclear.

**Thus, the hypothesis of the first aim of this thesis is that Rac1 is a critical regulator of cardiomyocyte polarity and development of RV myocardium and cardiac septum.**

The objectives of the first study were:

1. Generate a mouse line with an anterior SHF-specific Rac1 deficiency, $\text{Rac1}^{\text{SHF}}$.
2. Analyze $\text{Rac1}^{\text{SHF}}$ hearts for CHDs and defects in cardiomyocyte polarity.
3. Determine the role of Rac1 in SHF progenitors during embryonic heart development.

**1.5.2 Aim 2: Rac1 and Outflow Tract Development**

Development of the OFT seems to be especially dependent on cell polarity signaling mechanisms. Disturbances in PCP signaling in the SHF result in a spectrum of OFT defects, supporting a critical role for polarity in development of the OFT. In addition to PCP signaling, SHF progenitors in the dorsal pericardial wall are organized into a polarized epithelium. Disruption of SHF progenitor apical-basal polarity impacts proper development of the OFT. This suggests that Rac1 signaling in the SHF may have a crucial role in OFT development, given its known role in actin organization downstream of cell polarity signaling pathways. **Therefore, the hypothesis of the**
The second aim of this thesis is that Rac1 regulates SHF progenitor polarity and OFT development.

The objectives of the second study were:

1. Analyze $\text{Rac1}^{\text{SHF}}$ embryos for defects in SHF progenitor cell polarity.

2. Analyze $\text{Rac1}^{\text{SHF}}$ hearts for OFT defects.

3. To understand the mechanism by which Rac1 signaling in the SHF regulates OFT development.

1.5.3 Aim 3: Rac1 and Ventricular Myocardium Development

The developing ventricles have a high rate of proliferation and undergo a process of remodeling and compaction to form the trabeculae and compact myocardium. The mature myocardium is high organized with aligned, polarized cardiomyocytes. Multiple signaling pathways have been shown to play a role in ventricular myocardium development including Notch, BMP, FGF, retinoic acid and PCP signaling. However, the complete signaling mechanisms underlying ventricular myocardium development are still not completely understood. The known role of Rac1 in regulation of proliferation and cell organization suggests a role for Rac1 signaling in development of the ventricular myocardium. The $\text{Nkx2.5-Cre}$ transgenic mouse was used for the third study to elucidate the role of Rac1 in ventricular myocardium development. The Cre recombinase in the $\text{Nkx2.5-Cre}$ transgenic mouse is activated by E8.5. Therefore, the hypothesis of the third aim of this thesis is that Rac1 regulates proliferation and orientation of cardiomyocytes and development of the ventricular myocardium.
The objectives of the third study were:

1. To generate a ventricular myocardium specific Rac1 conditional knockout mouse ($Rac1^{Nkx2.5}$).

2. To analyze $Rac1^{Nkx2.5}$ hearts for CHDs and defects in proliferation and orientation of cardiomyocytes.

3. To understand the mechanisms by which Rac1 signaling regulates proliferation and organization of cardiomyocytes in the ventricular myocardium.
1.6 References


33. Erickson CA, Goins TL. Avian neural crest cells can migrate in the dorsolateral path only if they are specified as melanocytes. *Development*. 1995;121:915-924.


63. Snarr BS, Wirrig EE, Phelps AL, Trusk TC, Wessels A. A spatiotemporal evaluation of the contribution of the dorsal mesenchymal protrusion to cardiac development. *Dev Dyn*. 2007;236:1287-1294.


104. Pu WT, Ishiwata T, Juraszek AL, Ma Q, Izumo S. GATA4 is a dosage-sensitive regulator of cardiac morphogenesis. *Dev Biol.* 2004;275:235-244.


Chapter 2

Rac1 signaling is critical to cardiomyocyte polarity and embryonic heart development

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Carmen Leung, Xiangru Lu, Murong Liu, Qingping Feng

Departments of Physiology and Pharmacology (C.L., X.L, Q.F.), Medicine (Q.F.), Schulich School of Medicine and Dentistry, Collaborative Program in Developmental Biology (C.L.), Lawson Health Research Institute (M.L., Q.F.), The University of Western Ontario, London, Ontario, Canada N6A 5C1.

“Rac1 signaling is critical to cardiomyocyte polarity and embryonic heart development”
2 Chapter 2

2.1 Chapter Summary

Defects in cardiac septation are the most common form of congenital heart disease but the mechanisms underlying these defects are still poorly understood. The small GTPase Rac1 is implicated in planar cell polarity of epithelial cells in *Drosophila*. However, its role in mammalian cardiomyocyte polarity is not clear. Here, we tested the hypothesis that Rac1 signaling in the second heart field (SHF) regulates cardiomyocyte polarity, chamber septation and right ventricle (RV) development during embryonic heart development. Mice with SHF-specific deficiency of Rac1 (Rac1\textsubscript{SHF}) exhibited ventricular and atrial septal defects, a thinner RV myocardium and a bifid cardiac apex. Fate-mapping analysis showed that SHF contribution to the interventricular septum and RV was deficient in Rac1\textsubscript{SHF} hearts. Notably, cardiomyocytes had a spherical shape with disrupted F-actin filaments in Rac1\textsubscript{SHF} compared to elongated and well aligned cardiomyocytes in littermate controls. Expression of Scrib, a core protein in planar cell polarity, was lost in Rac1\textsubscript{SHF} hearts with decreased expression of WAVE and Arp2/3, leading to decreased migratory ability. Additionally, Rac1 deficient neonatal cardiomyocytes displayed defects in cell projections, lamellipodia formation and cell elongation. Furthermore, apoptosis was increased and the expression of *Gata4*, *Tbx5*, *Nkx2.5* and *Hand2* transcription factors was decreased in the Rac1\textsubscript{SHF} RV myocardium. Deficiency of Rac1 in the SHF impairs elongation and cytoskeleton organization of cardiomyocytes, and results in congenital septal defects, thin RV myocardium and a bifid cardiac apex. Our study suggests that Rac1 signaling is critical to cardiomyocyte polarity and embryonic heart development.
2.2 Introduction

The adult cardiomyocytes are highly polarized with a rod shape and the majority of their cell junction proteins localized to the intercalated discs, joining the cells end to end to facilitate electrical conduction and contraction of the heart. However, during embryonic development, cardiomyocytes are initially formed in spherical shapes. They begin to elongate at E13.5 and gradually assume a rod shape during fetal and postnatal development. The spatial differences or asymmetries in the shape, orientation and structure of cells define cell polarity, which is controlled by planar cell polarity (PCP) signalling, also known as the non-canonical Wnt pathway. Initially identified in Drosophila, the PCP pathway is well conserved in mammalian species. The ligands of this pathway include Wnt5a and Wnt11. The transmembrane receptors involved are Frizzled and Vangl2/Scrib. Through recruitment of Dishevelled, the PCP signals are transduced to the small GTPases RhoA and Rac, which in turn regulate actin polymerization and cell polarity. The important role of PCP signaling in regulating cardiomyocyte polarity and embryonic heart development has recently emerged. Genetic knockout or mutation of PCP components such as Wnt11, Vangl2, Scrib, and Dishevelled2 in mice all show congenital heart defects. Additionally, Wnt11/− and Vangl2 mutant mice show thin myocardium and defects in cardiomyocyte elongation, organization and migration. However, the role of Rac in cardiomyocyte polarity and embryonic heart development is unknown.

Rac GTPases are small (20-30 kDa) monomeric, signaling GTP-binding proteins, which are a subfamily of the Rho family of GTPases with four members: Rac1, Rac2, Rac3 and RhoG. Rac1 is a key molecule in the PCP pathway to promote cell polarity of the eyes and wings in Drosophila. Rac1 is also an important regulator of cell migration
and survival. Whole body Rac1−/− mice die before E9.5, with defects in germ layer formation due to reduced cell adhesion and motility and increased apoptosis within the mesoderm. Furthermore, Rac1−/− embryos fail to specify an anterior-posterior axis since cells in the anterior visceral endoderm do not migrate, suggesting an important role of Rac1 in cell polarity during embryogenesis.

The heart develops from three distinct populations of cells: the first heart field (FHF), second heart field (SHF) and cardiac neural crest. Initially, the primary heart tube is formed mainly from the FHF progenitors. SHF cells are then added to the heart tube to form the right ventricle and the outflow tract with contributions from the cardiac neural crest cells. Additionally, SHF progenitors are critical to the formation of the cardiac septum. Abnormalities in SHF development result in congenital heart defects (CHDs) in mice including septal defects, which are some of the most common types of CHDs in humans. To specifically study the role of Rac1 in cardiomyocyte polarity, right ventricle and cardiac septal development, we have generated a novel mouse model with a SHF (or anterior heart field, AHF) specific deficiency of Rac1 (Rac1SHF) using the Mef2c-Cre mouse, which directs Cre activity in the SHF. Our results show that Rac1 signaling in the SHF is critical to cardiomyocyte polarity, cardiac septation and RV development.

2.3 Methods

2.3.1 Mice

Rac1+/− C57BL/6 mice (Stock #5550), and C57BL/6 mT/mG mice (Stock #7676) were obtained from Jackson Laboratory (Bar Harbor, Maine). The mT/mG
mouse is a global double-fluorescent Cre reporter mouse, which expresses membrane-targeted Tomato (mT) before Cre excision and membrane-targeted GFP (mG) after excision of mT. The Mef2c-Cre embryos were obtained from Mutant Mouse Regional Resource Centers (MMRRC, Chapel Hill, North Carolina) and rederived. A breeding program was carried out to generate Mef2c-Cre;Rac1^f/f (Rac1^SHF), Mef2c-Cre;mT/mG and Rac1^SHF;mT/mG transgenic mice. Genotyping was performed by PCR using genomic DNA from tail biopsies. Primer sequences can be found in Table 2.1. All mouse experiments and procedures were carried out in accordance with the guidelines of the Canadian Council of Animal Care and approved by the Animal Use Subcommittee at the University of Western Ontario, Canada.

Table 2.1. The genotyping PCR primer sequences.

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<th>Reverse</th>
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<td>gatgtctaggggtgagcc</td>
</tr>
<tr>
<td>mTmG</td>
<td>ctgtgtgctctctgctct</td>
<td>cgagcggatcacaagcaata</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mutant reverse:</td>
</tr>
<tr>
<td></td>
<td></td>
<td>tcaatgggccccgggcttgttt</td>
</tr>
</tbody>
</table>

2.3.2 Histological Analysis

Neonatal and embryonic samples were fixed overnight in 4% paraformaldehyde at 4°C, dehydrated and paraffin embedded. Embryos were serially sectioned at 5 μm from
the top of the aortic arch to the apex with a Leica RM2255 microtome. Sections were mounted onto positively charged albumin/glycerin coated microslides in a set of five, with 25 µm intervals between each section. Slides were stained with hematoxylin and eosin (H/E) for histological analysis. Images were captured using a light microscope (Observer D1, Zeiss, Germany).

2.3.3 Immunohistochemistry

Immunohistochemical staining was performed on heart sections (5 µm). Antigen retrieval was carried out in sodium citrate buffer (pH 6.0) at 92 °C using a BP-111 laboratory microwave (Microwave Research & Applications, Carol Stream, Illinois). Immunostaining was performed with primary antibodies for Rac1 (Santa Cruz), phosphohistone-H3 (phospho S10) (Abcam), cleaved caspase-3 (Cell Signaling), active (non-phosphorylated) β-catenin (Cell Signaling) and GFP (Abcam). All slides were imaged with Zeiss Observer D1 microscope using AxioVision Rel 4.7 software. For phalloidin and wheat germ agglutinin (WGA) staining, P0 heart samples were fixed in 4% paraformaldehyde, cryoprotected in 30% sucrose, embedded in FSC22 frozen section media (Leica) and sectioned with a Leica cryostat at 10 µm thick onto glass slides. Slides were incubated with Alexa Fluor 488 phalloidin (Life Technologies), Alexa Fluor 647 WGA (Invitrogen) and Hoechst 33342 (Invitrogen). Confocal images were obtained with a Zeiss LSM 510 Duo microscope using ZEN 2012 software (Zeiss, Germany).

2.3.4 Western Blot Analysis

Rac1 protein expression from P0 RV was measured by western blot analysis. Briefly, 40 µg of protein from isolated RV tissue was separated by 12% SDS-Page gel and transferred to nitrocellulose membranes. Blots were probed with antibodies against
Rac1 (1:500, Santa Cruz) and GAPDH (1:3000, Santa Cruz). Blots were then washed and probed with horseradish peroxidase conjugated secondary antibodies (1:2500, Bio-Rad) and detected using an ECL detection method. Signal quantification was performed by densitometry.

2.3.5 RV Explant Culture

Embryos were harvested at E12.5 and embryonic hearts were dissected to separate RV tissue from the whole heart. RV tissues were cultured on 1% collagen-coated tissue culture plastic multiwell plates, as previously described with the following adaptations. Explants were incubated for six days at 37°C with 5% CO₂ in Dulbecco’s modified eagle medium (DMEM) containing 10% FBS and 1% penicillin/streptomycin. Media was refreshed every two days.

2.3.6 Quantitative Real-Time RT-PCR

Total RNA was isolated from E13.5 RV using the RNeasy Mini Kit (QIAGEN). Reverse transcription reaction was performed using M-MLV Reverse Transcriptase (Invitrogen) and EvaGreen qPCR Mastermix (Applied Biological Materials Inc.) was used for real time thermal cycling. Samples were amplified for 35 cycles using the Eppendorf Mastercycler Realplex Real-Time PCR machine. 28S rRNA was used as an internal control. Primer sequences can be found in Table 2.2. The mRNA levels in relation to 28S rRNA were determined using a comparative C₅₀ method.
## Table 2.2. The real-time RT-PCR primer sequences.

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<th>Reverse</th>
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<td>ttcatagtccacaggaggaggtg</td>
</tr>
<tr>
<td><em>WAVE2</em></td>
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<td>cagagaatgaaggggaaggtgag</td>
</tr>
<tr>
<td><em>Nkx2.5</em></td>
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<td>gacgccaaagttcacaaggttct</td>
</tr>
<tr>
<td><em>Gata4</em></td>
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<td>gcctgcagtggtctgagtgac</td>
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<tr>
<td><em>Tbx5</em></td>
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</tr>
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<td><em>Rac1</em></td>
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<td>ttgctccagctgtgcccata</td>
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<tr>
<td><em>Tbx20</em></td>
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<td>gtcgctatggagctgtgtactgtggt</td>
</tr>
<tr>
<td><em>Hand2</em></td>
<td>gctacatcgctactctaggtg</td>
<td>tctgtgctgtgctgtcactgtt</td>
</tr>
<tr>
<td><em>28S</em></td>
<td>tgaatccgagggaggagag</td>
<td>acattgctcacaagtcagccag</td>
</tr>
</tbody>
</table>

### 2.3.7 Scratch Assay

Wild-type neonatal cardiomyocytes were prepared and cultured as previously described. Cardiomyocytes were infected with adenoviruses expressing a dominant-negative form of Rac1 (Ad-Rac1N17, Vector Biolabs, Philadelphia, PA, USA) or β-
galactosidase (Ad-β-gal, Vector Biolabs) as a control at a multiplicity of infection of 10 plaque forming units/cell. The scratch was performed 6 hours after adenoviral infection. Cells were fixed and immunostained with primary antibodies for Rac1 (Santa Cruz) and α-actinin (Sigma) 24 hours after scratch.

2.3.8 Statistical Analysis

Data are means ± SEM. Unpaired Student’s t test was used when data passed a normality test. Nonparametric Mann-Whitney test was employed when samples size was 3-4 per group. Differences were considered significant at $P \leq 0.05$.

2.4 Results

2.4.1 Generation of a transgenic mouse with Rac1 knockdown in the SHF

To elucidate the role of Rac1 in the SHF, the Mef2c-Cre mouse was crossed to the Rac1$^{f/f}$ mouse to generate Mef2c-Cre;Rac1$^{f/f}$ (Rac1$^{SHF}$) offspring. Rac1$^{SHF}$ mice have Rac1 gene specifically knocked down in SHF progenitors and their derivatives. Embryonic samples were collected and loss of the Rac1 transcript was confirmed in Rac1$^{SHF}$ hearts by quantitative RT-PCR. Rac1 mRNA expression was reduced by 51% in the RV myocardium of E13.5 Rac1$^{SHF}$ compared to littermate Rac1$^{f/f}$ controls (Fig. 2.1A). Immunostaining for Rac1 protein was also performed to further confirm the loss of myocardial Rac1 expression in SHF derivatives. In P0 Rac1$^{SHF}$ hearts, robust staining for Rac1 was observed in the LV. In the same P0 Rac1$^{SHF}$ heart section, the Rac1 signal intensity was markedly decreased in the RV when compared to the LV (Fig. 2.1B). Ratio of Rac1 to GAPDH protein was reduced by 40% in P0 Rac1$^{SHF}$ RV compared to
littermate Rac1<sup>ff</sup> controls as determined by Western blot analysis (Fig. 2.1C). These data show that the Mef2c-Cre mediated recombination downregulates Rac1 expression in SHF derivatives of the embryonic heart.
**Figure 2.1. Confirmation of Rac1 deletion in Rac1<sup>SHF</sup> hearts.**

(A) Rac1 mRNA levels were significantly decreased in Rac1<sup>SHF</sup> RV compared to Rac1<sup>f/f</sup> controls at E13.5, as measured by quantitative RT-PCR. **P<0.01 by unpaired Student’s t test. (B) Immunostaining shows that Rac1 protein levels were reduced in P0 Rac1<sup>SHF</sup> RV compared to the LV myocardium. (C) Western blot analysis of P0 RV showed a significant decrease in Rac1 protein in Rac1<sup>SHF</sup> compared to Rac1<sup>f/f</sup> controls. *P<0.05 by Mann-Whitney test. Scale bars: 100 µm (B, top panel), 20 µm (B, lower panels).

### 2.4.2 Deficiency of Rac1 in the SHF results in congenital heart defects

The most prominent defect upon initial analysis of P0 Rac1<sup>SHF</sup> hearts was a gross morphological abnormality in the overall shape of the heart. Instead of a distinct apex, all P0 Rac1<sup>SHF</sup> hearts (28/28) had a deep interventricular groove and bifurcation of the two ventricles, resulting in a bifid cardiac apex, a rare CHD in humans (Table 2.3, Fig. 2.2A, B). Atrial septal defects (ASD) were observed in 82% (23/28) of Rac1<sup>SHF</sup> hearts but not in littermate control Rac1<sup>f/f</sup> P0 hearts (0/19) (Table 2.3, Fig. 2.2C, D). Ventricular septal defects (VSD) were seen in 100% (28/28) of P0 Rac1<sup>SHF</sup> hearts (Table 2.3, Fig. 2.2C, D). Additionally, the RV myocardium had poor trabeculation and the compact myocardium was significantly thinner in Rac1<sup>SHF</sup> hearts compared to littermate controls (Fig. 2.2E-G and Table 2.3). These findings implicate a critical role for SHF Rac1 signaling in cardiac septation and RV development.
Figure 2.2. Mef2c-Cre induced Rac1 deletion in SHF results in congenital heart defects.

(A, B) Bifurcation between RV and LV (arrow) in P0 Rac1^{SHF} hearts. (C, D) Atrial septal defect (arrow head) and ventricular septal defect (arrow) were found in P0 Rac1^{SHF} hearts. (E-G) P0 Rac1^{SHF} RV compact myocardium (double arrows) was significantly thinner. ***P<0.001 by unpaired Student’s t test. RA, right atrium; LA, left atrium; RV, right ventricle; LV, left ventricle. Scale bars: 100 µm (C, D), 50 µm (E, F).
Table 2.3. Incidence of congenital heart defects in P0 \textit{Rac1}^{SHF} hearts.

\begin{tabular}{lccc}
 & \textbf{Rac1}^{ef} & \textbf{Mef2c-Cre;Rac1}^{f+} & \textbf{Rac1}^{SHF} \\
 & (n=19) & (n=10) & (n=28)* \\
\hline
Normal & 19 & 100 & 10 & 100 & 0 & 0 \\
ASD & 0 & 0 & 0 & 0 & 23 & 82 \\
VSD & 0 & 0 & 0 & 0 & 28 & 100 \\
Bifid apex & 0 & 0 & 0 & 0 & 28 & 100 \\
Defective RV & 0 & 0 & 0 & 0 & 28 & 100 \\
\end{tabular}

ASD, atrial septal defect; VSD, ventricular septal defect; RV, right ventricle. *21 alive and 7 collected dead at P0. Since penetrance of congenital heart defects is 100% in \textit{Rac1}^{SHF} hearts, statistical analysis is not necessary.

2.4.3 \textit{Rac1}^{SHF} hearts exhibit defective cardiomyocyte polarity

Cardiomyocyte organization and alignment were severely disrupted in P0 \textit{Rac1}^{SHF} hearts. Cell membrane staining with wheat germ agglutinin (WGA) revealed rounded, spherical shaped cardiomyocytes in \textit{Rac1}^{SHF} RV (Fig. 2.3B) compared to \textit{Rac1}^{ef} RV, which had elongated cardiomyocytes (Fig. 2.3A). Actin cytoskeleton organization was also severely disrupted in \textit{Rac1}^{SHF} RV, as shown by phalloidin staining to mark F-actin filaments (Fig. 2.3D). \textit{Rac1}^{SHF} RV had an absence of long F-actin filaments while \textit{Rac1}^{ef} RV myocardium exhibited long, parallel running F-actin fibers (Fig. 2.3C). In addition,
active (non-phosphorylated) β-catenin staining between cell junctions was also reduced in E13.5 Rac1SHF RV compared to Rac1ff RV (Fig. 2.3E and F). Overall, these data strongly implicates disruption of cardiomyocyte polarity in Rac1SHF hearts.
Figure 2.3. \( \text{Rac1}^{\text{SHF}} \) hearts exhibit defective polarity.

\( \text{(A, B)} \) Wheat germ agglutinin (WGA) staining to mark cell membranes of P0 \( \text{Rac1}^{\text{SHF}} \) RV shows rounded cardiomyocytes compared to the elongated cells of littermate controls. \( \text{(C, D)} \) Phalloidin staining to mark F-actin shows disorganization in cellular structure and a deficiency in long actin filaments in the \( \text{Rac1}^{\text{SHF}} \) RV. \( \text{(E, F)} \) Active (non-phosphorylated) \( \beta \)-catenin staining is reduced at cell-cell junctions in E13.5 \( \text{Rac1}^{\text{SHF}} \) RV. White arrows indicate cell adhesion. Scale bars: 20 \( \mu \text{m} \) (A-D), 10 \( \mu \text{m} \) (E, F).

2.4.4 Expression of Scrib protein is lost in \( \text{Rac1}^{\text{SHF}} \) hearts

Scrib, the mouse homolog of the \textit{Drosophila} protein Scribble, is a component of the PCP pathway. Immunostaining shows Scrib protein was expressed mainly in the lower part of RV and the cardiac apex in \( \text{Rac1}^{\text{f/f}} \) hearts (Fig. 2.4A-B, E-F), and the number of Scrib\(^+\) cells was about 10 times higher in E15.5 than E12.5 \( \text{Rac1}^{\text{f/f}} \) hearts (Fig. 2.4I-J). Notably, Scrib expression was almost absent in both E12.5 and E15.5 \( \text{Rac1}^{\text{SHF}} \) hearts (Fig. 2.4C-D, G-H) and the number of Scrib\(^+\) cells was significantly decreased in \( \text{Rac1}^{\text{SHF}} \) hearts compared to littermate controls (Fig. 2.4I-J). The loss of Scrib expression implicates disruption of the PCP pathway and further supports a failure to establish cardiomyocyte polarity in \( \text{Rac1}^{\text{SHF}} \) hearts.
Figure 2.4. Decreased Scrib protein expression in Racl^{SHF} hearts.

(A-D) Scrib immunostaining was performed on E12.5 paraffin sections. Arrows indicate Scrib protein expression. B and D are magnifications of box in A and C, respectively. (E-H) Scrib immunostaining was performed on E15.5 paraffin sections. Arrows indicate Scrib protein expression. F and H are magnifications of box in E and G, respectively. (I, J) Number of Scrib positive cells was significantly decreased in E12.5 and E15.5 Racl^{SHF} hearts. n=3 hearts per group, *P=0.05 by Mann-Whitney test. Scale bars: 100 µm (A, C, E, G), 20 µm (B, D, F, H).

2.4.5 Racl is required for lamellipodia formation and cardiomyocyte migration

Cell migration is a fundamental process of gastrulation, morphogenesis and formation of organs and tissues during development. To determine whether Racl plays a migratory role in SHF derived-cells, RV from E12.5 Racl^{SHF};mTmG and control Mef2c-Cre;Racl^f/+;mTmG control hearts were isolated and explants cultured on collagen gels for six days. The mTmG global double-fluorescent Cre reporter mouse expresses membrane-targeted Tomato (mT) before Cre-mediated excision and membrane-targeted GFP (mG) after excision of mT. Thus all SHF-derived cells in Racl^{SHF};mTmG and Mef2c-Cre;Racl^f/+;mTmG are GFP^+. It was observed that Mef2c-Cre; Racl^f/+;mTmG control explants had both non-SHF derived (mT - labeled) and SHF-derived (mG - labeled) cells migrating out from the RV explant by day six (Fig. 2.5A-C). In contrast, Racl^{SHF};mTmG explants only had non-SHF derived cells (mT - labeled) migrating out from the RV explant and very little to no SHF-derived (mG - labeled) cell migration (Fig. 2.5D-F). The migration defects observed in the Racl^{SHF};mTmG RV explants indicate that
Rac1 regulates migration of SHF-derived progenitor cells, likely through actin organization controlled by the WAVE and Arp2/3 pathway downstream of Rac1. A significant decrease was found in Abi1, WAVE2, Arp2 and Arp3 expression, components of the WAVE and Arp2/3 complex, in E13.5 Rac1<sup>SHF</sup> RV compared to littermate controls (Table 2.4). To further assess cell migration, scratch assays were performed on neonatal cardiomyocytes infected with adenoviral constructs of β-galactosidase (Ad-βgal) or dominant negative Rac1 (Ad-Rac1N17). It was observed that 24 hours after scratch, Ad-βgal-treated cardiomyocytes were aligned perpendicular to the scratch edge and had projections along the scratch edge, indicative of actin organization and lamellipodia extension (Fig. 2.5G). In contrast, no projections and lamellipodia formation were observed in Ad-Rac1N17 cardiomyocytes and the cells were disorganized and arranged in a random orientation along the scratch edge (Fig. 2.5H). Overall, these findings implicate Rac1 in the regulation of cell migration and lamellipodia extension in cardiomyocytes.
Figure 2.5. Rac1 deficiency results in defective cell migration and lamellipodia formation.

(A-C) E12.5 RV explant cultures of Mef2c-Cre;mTmG;Racl<sup>F</sup> hearts have both SHF-derived (mG) and non-SHF-derived (mT) migrating cells. (E-F) E12.5 RV explant cultures of Rac1<sup>SHF</sup>;mTmG hearts have only non-SHF-derived (mT) migrating cells without any SHF-derived migrating cells (mG). (G-H) Scratch assays of adenoviral
infected neonatal cardiomyocytes show cellular projections and lamellipodia extensions (arrows) beyond the scratch edge in Ad-βgal treated cells and none in Ad-Rac1N17 treated cells 24 hours post-scratch. Dotted white line indicates scratch edge. Shown are representative images from 3 independent experiments.

Table 2.4. Real-time PCR analysis of gene mRNA levels in E13.5 Rac1^{SHF} RV.

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<tr>
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<th>Rac1^{SHF}</th>
<th>P Values</th>
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<td>0.0462 ± 0.00467 (n=6)</td>
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<td>Gata4</td>
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<td>Hand2</td>
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</tbody>
</table>

Data are ratios to 28S and presented as mean ± SEM. Significance in gene expression difference between Rac1^{SHF} and Rac1^{ff} littermates analyzed by unpaired Student’s t test. P<0.05 was considered statistically significant. No significant differences were found in Tbx20 and β-actin mRNA expression.
2.4.6 *Rac1<sup>SHF</sup> cardiomyocytes are shortened and rounded*

Neonatal cardiomyocyte cultures from *Rac1<sup>SHF</sup> RV* tissues showed that these cells had disrupted actin organization as indicated by phalloidin staining and the absence of distinct cell projections compared to *Rac1<sup>f/f</sup>* cardiomyocytes. Interestingly, patterning of α-actinin seemed to be disrupted as well in *Rac1<sup>SHF</sup>* cardiomyocytes (*Fig. 2.6A, B*). Measurement of cardiomyocyte long axis length revealed significantly shorter *Rac1<sup>SHF</sup>* cardiomyocytes compared to littermate control cardiomyocytes (*Fig. 2.6E*). Additionally, neonatal *Rac1<sup>SHF</sup>* cardiomyocytes were rounded compared to control *Rac1<sup>f/f</sup>* cardiomyocytes, which had an elongated morphology (*Fig. 2.6C, D*). No rounded cardiomyocytes were found in *Rac1<sup>f/f</sup>* cultures while over 40% of *Rac1<sup>SHF</sup>* cardiomyocytes exhibited a rounded morphology (*Fig. 2.6F*). These data strongly support the role of Rac1 in regulating elongation and polarization of cardiomyocytes.
Figure 2.6. Cells are rounded and cell length is reduced in cultured neonatal  
\textit{Rac1^{SHF}} cardiomyocytes.

\textbf{(A, B)} Phalloidin and \(\alpha\)-actinin staining of neonatal cardiomyocytes show defective cellular organization and projection formation in \textit{Rac1^{SHF}} cells. \textbf{(C, D)} \textit{Rac1^{SHF}} cardiomyocytes are a spherical shape compared to \textit{Rac1^{ff}} controls. \textbf{(E, F)} Long axis measurements of \textit{Rac1^{SHF}} cardiomyocytes showed that these cells are significantly shorter and are more spherical compared to \textit{Rac1^{ff}} controls. \(n=3\) independent cultures per group, * \(P=0.05\) by Mann-Whitney test (E). Since penetrance of round cardiomyocyte is 0\% in \textit{Rac1^{ff}} cultures, statistical analysis is not necessary (F). Scale bars: 10 \(\mu\)m (A, B), 20 \(\mu\)m (C, D).

2.4.7 Contribution of SHF progenitors to \textit{Rac1^{SHF}} hearts is decreased

Histological analysis showed that a deep fissure between the RV and LV was observed in E11.5 \textit{Rac1^{SHF}} hearts while this was not evident and the early muscular interventricular septum was beginning to form in littermate control hearts (Fig. 2.7A, B). At E12.5, bifurcation of the RV and LV in \textit{Rac1^{SHF}} hearts continued to be present (Fig. 2.7C, D) and by E15.5 a distinct apex was formed in control hearts while \textit{Rac1^{SHF}} hearts continued to have a deep cleft separating the ventricles (Fig. 2.7E, F). Fate-mapping with the \textit{mT/mG} global double-fluorescent Cre reporter mouse\textsuperscript{18} was performed to follow the developmental progression of the SHF progenitors. A deficiency in the SHF lineage contributing to the developing E11.5 \textit{Rac1^{SHF};mTmG} hearts was evident, especially in the early forming muscular interventricular septum between the RV and LV (Fig. 2.7G, H). At E12.5 and E15.5, the interventricular septum in \textit{Rac1^{SHF};mT/mG} hearts had a major deficiency of SHF-derived cells, leading to formation of a bifid apex (Fig. 2.7I-L). These
findings implicate a critical role of SHF Rac1 signaling in muscular interventricular septum formation.

**Figure 2.7. Deficient SHF progenitor contribution to Rac1<sup>S</sup>HF hearts.**

(A-F) H/E staining of E11.5, E12.5 and E15.5 heart sections shows defective interventricular septum formation (arrow) in Rac1<sup>S</sup>HF hearts, leading to a bifid cardiac apex. (G-L) Fate mapping with mT/mG reporter shows a decreased SHF progenitor contribution to the interventricular septum (arrow) in Rac1<sup>S</sup>HF hearts. Paraffin sections were immunostained with anti-GFP. Scale bars: 100 µm (A-J), 200 µm (K, L).
2.4.8 Rac1 promotes cell survival in SHF-derived myocardium

Aberrant apoptosis or decreased proliferation in the SHF may contribute to CHDs. To determine whether an abnormality in proliferation was a factor in Rac1<sup>SHF</sup> hearts, phosphohistone-H3 (pHH3) protein staining was performed on E11.5 heart sections (Fig. 2.8A-F). No differences in proliferation were observed between Rac1<sup>SHF</sup> and Rac1<sup>f/f</sup> control hearts (Fig. 2.8G). Next, we stained for cleaved caspase-3 (the active form of caspase-3) on E11.5 heart sections to detect cell apoptosis (Fig. 2.9A-D). Cleaved caspase-3 staining was quantified as a percentage to the total number of cells. E11.5 Rac1<sup>SHF</sup> hearts had significantly higher levels of apoptosis in the RV compared to littermate controls (Fig. 2.9E, *P*<0.05). Similarly, increased apoptosis was also observed in the interventricular septum of E11.5 Rac1<sup>SHF</sup> hearts (Fig. 2.9B, D, E, *P*<0.05). No significant difference in apoptosis was observed in the LV between E11.5 Rac1<sup>SHF</sup> and control hearts (Fig. 2.9E, *P*=0.27). Together, this data defines a requirement for Rac1 in cell survival but not proliferation in the development of SHF-derived cardiac structures.
Figure 2.8. Phospho histone H3 (pHH3) protein staining to assess cell proliferation in E11.5 $Rac1^{SHF}$ hearts.

(A-F) Representative pHH3 staining in transverse heart sections. Arrows indicate positive signals. Scale bars: 20 µm. (G) No significant differences in cell proliferation were found between E11.5 $Rac1^{SHF}$ and $Rac1^{ff}$ hearts. n=3 hearts per group.
Figure 2.9. Increased apoptosis in Rac1$^{SHF}$ RV and interventricular septum.

(A-D) E11.5 heart sections were immunostained with cleaved caspase-3 antibody to mark apoptotic cells. Arrows indicate positive signals. B and D are magnifications of the box in A and C, respectively. (E) Quantification of apoptosis in RV, LV and the cardiac septum. * $P<0.05$ vs. corresponding Rac1$^{ff}$ by Mann-Whitney test. Scale bars: 100 µm (A, C), 10 µm (B, D). (F) The proposed pathway of Rac1 signaling in SHF derivatives during embryonic heart development. Rac1 regulates actin organization to promote cardiomyocyte polarity and cell migration. Additionally, Rac1 promotes cell survival and the expression of cardiac developmental genes. Overall, these effects of Rac1 contribute to normal RV and septal development of the heart.

2.4.9 Downregulation of transcription factors

Heart development is regulated by a complex network of transcription factors, which interact synergistically and in a dose-dependent manner to activate target genes.$^{24}$ To address the genetic pathways regulated by Rac1 signaling in the SHF, we analyzed known transcription factors important for heart development. Expression levels of Gata4, Nkx2.5 and Tbx5 mRNA were significantly decreased in the RV of E13.5 Rac1$^{SHF}$ compared to Rac1$^{ff}$ hearts (Table 2.4). Hand2 and Tbx20 are two transcription factors shown to be expressed predominantly in the RV and regulate RV development.$^{25, 26}$ Hand2 expression was significantly decreased in the RV of E13.5 Rac1$^{SHF}$ compared to Rac1$^{ff}$ hearts (Table 2.4). However, no significant differences were found in Tbx20 expression between Rac1$^{ff}$ and Rac1$^{SHF}$ RV (Table 2.4). Taken together, these findings indicate that transcriptional regulation of RV development was severely disrupted in the Rac1$^{SHF}$ hearts.
2.5 Discussion

The present study was carried out to examine the role of Rac1 in SHF progenitors. Using the SHF specific Mef2c-Cre mouse line to delete Rac1 in SHF progenitors and derivatives, we demonstrated that a Rac1 deficiency in the SHF leads to 100% penetrance of CHDs including septal defects, thin RV myocardium and defective trabeculation and most notably, a bifid cardiac apex. Our data showed for the first time that Rac1 signaling is critical to cardiomyocyte polarity and embryonic heart development with contributions of cell migration, survival and cardiac gene expression (Fig. 2.9F).

Rac1 has been shown to be a downstream effector of the PCP pathway by modulating the cytoskeleton and regulating actin dynamics in Drosophila and Xenopus. However, the role of Rac1 in mammalian cardiomyocyte polarity and heart development is not known. Studies have shown that disruption of the PCP pathway in mice by deletion of Scrib resulted in cellular disorganization, defective trabeculation, thin myocardium and chamber septation defects, which are strikingly similar to the defects observed in this study and strongly supports a disruption of PCP signaling in Rac1SHF hearts. Interestingly, we observed that Scrib protein expression pattern is most abundant in the RV and interventricular septum junction during embryonic heart development. Notably, Scrib protein expression in this distinct cardiac tissue area is lost in Rac1SHF hearts. We postulate that cardiomyocytes at the RV and interventricular junction must be highly polarized and oriented to align with the forming cardiac apex. The rounded, non-polarized and disorganized Rac1SHF cardiomyocytes are likely unable to bridge the RV and interventricular junction to unify the two ventricles to form a cardiac apex resulting in bifurcation of the muscular septum. In addition, a decrease in cell number due to
increase in apoptosis in this area may also contribute to the development of bifid apex. It has been reported that genetic deletion of transcription factor \textit{Ets1} also results in VSD and bifid cardiac apex.\textsuperscript{28} However, another group using the same \textit{Ets1} knockout mouse did not observe a bifid cardiac apex.\textsuperscript{29} This makes the role of \textit{Ets1} still unclear in the formation of a bifid cardiac apex. Recorded cases of bifid cardiac apex in humans are very rare and have usually been reported concomitantly with other congenital heart anomalies including ASD, VSD and defective RV development,\textsuperscript{30, 31} which are very similar to the type of defects that were observed in \textit{Rac1}\textsubscript{SHF} hearts. Thus, it is possible that the bifid cardiac apex and associated cardiac defects found in humans could be partially associated with abnormalities in \textit{Rac1} signaling and defects in cardiomyocyte polarity during embryonic heart development.

It is well established that actin is the most abundant cytoskeleton protein and plays a critical role in controlling cell orientation, cell shape and migration. \textit{Rac1} is an important regulator of actin polymerization, ruffle formation, lamellipodia extension and migration in numerous cell types.\textsuperscript{7} \textit{Rac1} is known to activate the Wiskott-Aldrich syndrome protein (WASP)-family verprolin-homologous protein (WAVE) complex, which is recruited to the plasma membrane and regulates the actin cytoskeleton through the actin-related protein-2/3 (ARP2/3) complex. ARP2/3 associates with actin filaments to promote formation of new filaments from the sides of existing filaments, creating a dendritic network of branched actin filaments to extend lamellipodia and propel cells forward during migration.\textsuperscript{32, 33} The combined defects in cell polarity, morphology, lamellipodia formation and actin organization observed in SHF-derived cells led to an overall inhibition of cardiomyocyte migratory ability in \textit{Rac1}\textsubscript{SHF} embryos. Furthermore,
Rac1 has been shown to promote cell survival by activation of the PI3K/ Akt pathway.\textsuperscript{34-36} Decreased migration ability and an increased rate of apoptosis in SHF-derived cells likely concomitantly contribute to an overall deficiency in SHF contribution to the developing Rac1\textsuperscript{SHF} heart.

Numerous studies have found that Rac1 can regulate gene expression through different pathways. Interestingly, it has been shown that Rac1 can activate the canonical Wnt pathway by promoting nuclear accumulation of β-catenin, the effector of the Wnt pathway.\textsuperscript{37,38} Ai \textit{et al.} showed that deletion of β-catenin in the SHF led to abnormalities in development of the RV and interventricular septum,\textsuperscript{39} very similar to the defects observed in Rac1\textsuperscript{SHF} mice in the present study. However, whether the defects were also due to loss of β-catenin cell-cell adhesion was not addressed in their study. Our data showed a loss of β-catenin cell adhesions in the Rac1\textsuperscript{SHF} RV myocardium, supporting a defect in polarity and places non-canonical Wnt signaling as the main driver of the cellular and morphological defects observed. Our data is supported by Abhul-Ghani \textit{et al}, where they showed that non-canonical Wnt, which is highly active during cardiogenesis, antagonizes the canonical Wnt pathway.\textsuperscript{40} However, the contribution of canonical Wnt signaling cannot be ruled out in our study since it has been shown to play a role in specification and expansion of early SHF progenitors.\textsuperscript{41} Rac1 can also promote gene expression through NF-κB and JNK pathways.\textsuperscript{42,43} Our data showed that Rac1\textsuperscript{SHF} hearts have decreased expression of \textit{Gata4}, \textit{Tbx5}, \textit{Nkx2.5} and \textit{Hand2} transcription factors in the developing RV. Thus, it is plausible that a Rac1 deficiency in the SHF will lead to decreased NF-κB and/or JNK signaling and down-regulation of key cardiac transcription factors during embryonic heart development. \textit{Tbx20} acts in a dose-sensitive manner as an
essential regulator of outflow tract, cardiac valve and RV development.\textsuperscript{26} However, no significant down-regulation of \textit{Tbx20} expression was found in the \textit{Rac1}\textsubscript{SHF} hearts. A hierarchy of distinct cardiac transcription factor regulation exists in the developing heart.\textsuperscript{44, 45} Our data suggests that Rac1 regulates a distinct network of cardiac transcription factors that do not include \textit{Tbx20} in SHF-derivatives. Future studies will be necessary to determine what exact downstream pathways Rac1 regulates in the SHF to promote cardiac gene expression.

\textit{Tbx5} is essential in the SHF for atrial septation,\textsuperscript{46} despite it is predominately expressed in the LV during early heart development.\textsuperscript{47, 48} In addition, Später \textit{et al} recently challenged the view of \textit{Tbx5} as a classical FHF marker,\textsuperscript{49} where they showed an overlap of expression pattern between the posterior end of the \textit{Tbx5}-positive FHF domain and the \textit{Isl1}-positive SHF domain, thus implicating a role for \textit{Tbx5} in the SHF. Our data is compatible with the studies on \textit{Tbx5} from Nadeau \textit{et al},\textsuperscript{50} and Xie \textit{et al},\textsuperscript{46} where they showed that Gata4 and \textit{Tbx5} co-operatively regulate atrial septum development and haploinsufficiency of \textit{Tbx5} in the SHF results in ASDs.

Although further investigations are required to analyze potential \textit{RAC1} gene mutations in humans with bifid cardiac apex, the present study has shown that SHF \textit{Rac1} is critical to septal and RV development and deficiencies in SHF Rac1 can give rise to bifid cardiac apex along with the associated CHDs. Our data is the first to show Rac1 as an essential regulator of cardiomyocyte polarity during heart development and to provide a possible mechanism for how bifid cardiac apex arises. Establishment of cellular polarity is essential for the processes regulating cell morphology, lamellipodia formation and cell
migration in the developing embryo and this seems to be especially critical in formation of a distinct cardiac apex during embryonic heart development.

2.6 Footnotes

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2.7 References


Chapter 3

Rac1 signaling is required for anterior second heart field cellular organization and cardiac outflow tract development

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Carmen Leung, Yin Liu, Xiangru Lu, Mella Kim, Thomas A. Drysdale, Qingping Feng

Departments of Physiology and Pharmacology, Medicine (Q.F.), Schulich School of Medicine and Dentistry, Collaborative Program in Developmental Biology (C.L.), Children’s Health Research Institute (T.A.D., Q.F.), Lawson Health Research Institute (Q.F.), The University of Western Ontario, London, Ontario, Canada N6A 5C1.

“Rac1 signaling is required for anterior second heart field cellular organization and cardiac outflow tract development”
Chapter 3

3.1 Chapter Summary

The small GTPase Rac1 regulates diverse cellular functions, including both apicobasal and planar cell polarity (PCP) pathways. However, its role in cardiac outflow tract (OFT) development remains unknown. In the present study, we aimed to examine the role of Rac1 in the anterior second heart field (SHF) splanchnic mesoderm and subsequent OFT development during heart morphogenesis. Using the Cre/loxP system, mice with an anterior SHF-specific deletion of Rac1 (Rac1\textsuperscript{SHF}) were generated. Embryos were collected at various developmental time points for immunostaining and histological analysis. Intrauterine echocardiography was also performed to assess aortic valve blood flow in E18.5 embryos. The Rac1\textsuperscript{SHF} splanchnic mesoderm exhibited disruptions in SHF progenitor cellular organization and proliferation. Consequently, this led to a spectrum of OFT defects along with aortic valve defects in Rac1\textsuperscript{SHF} embryos. Mechanistically, it was found that the ability of the Rac1\textsuperscript{SHF} OFT myocardial cells to migrate into the proximal OFT cushion was severely reduced. In addition, expression of the neural crest chemoattractant semaphorin, Sema3c, was decreased. Lineage tracing showed that anterior SHF contribution to the OFT myocardium and aortic valves was deficient in Rac1\textsuperscript{SHF} hearts. Furthermore, functional analysis with intrauterine echocardiography at E18.5 showed aortic valve regurgitation in Rac1\textsuperscript{SHF} hearts, which was not seen in control hearts. Disruptions to Rac1 signaling in the anterior SHF results in aberrant progenitor cellular organization and defects in OFT development. Our data show Rac1 signaling as a critical regulator of cardiac OFT formation during embryonic heart development.
3.2 Introduction

Cardiac outflow tract (OFT) defects account for approximately one third of all congenital heart defects (CHDs) reported in human births and often requires intervention within the first year of life. Even after surgical correction, risk of morbidity and mortality from OFT defects remains high.\textsuperscript{1,2} However, the molecular mechanisms underlying OFT defects are not well defined. Understanding the developmental mechanisms of OFT formation is crucial for new insights into improving diagnostics and designing therapeutic approaches for CHD patients.

Cell polarity is the asymmetrical organization of cell membrane proteins, intracellular organelles and actin cytoskeleton that can influence cell fate and specialized functions such as migration and proliferation.\textsuperscript{3} Establishment of polarity is a critical step in a multitude of developmental events, including formation of the OFT.\textsuperscript{4,5} The intricate process of OFT development involves coordination and interactions between two distinct cell types, second heart field (SHF) progenitors and cardiac neural crest cells.\textsuperscript{6} The SHF progenitors give rise to the myocardial and endothelial cells of the OFT, semilunar valves, along with vascular smooth muscle cells at the base of the aorta and pulmonary trunk. Neural crest cells contribute to septation of the OFT and remodeling of the aortic arches and semilunar valves.\textsuperscript{7,8} At approximately E8.5, cardiac neural crest cells will delaminate from the neural tube, migrate into the pharyngeal region and become closely apposed with SHF progenitors in the dorsal pericardial wall.\textsuperscript{9} SHF cells form an apicobasally-polarized epithelium in the splanchnic mesoderm and maintenance of this apicobasal polarity is crucial for heart tube elongation and OFT morphogenesis.\textsuperscript{10} Planar cell polarity (PCP), which is orthogonal to apicobasal polarity, has also been shown to be
a critical regulator of the SHF as tissue-specific deletion of core PCP genes, *Vangl2* and *Dishevelled1/2*, recapitulates the spectrum of OFT defects reported in the full-body mutants.\(^{11,12}\) Recent studies have suggested that apicobasal polarity and PCP signaling are interconnected where crosstalk occurs to maintain cellular polarity and overall tissue architecture.\(^{13,14}\)

Rac1 is a small signaling GTPase from the Rac subfamily of Rho GTPases and has been implicated in both apicobasal polarity and PCP signaling pathways.\(^{15,16}\) Rac1 acts as a pleiotropic effector of numerous cellular processes including regulation of the actin cytoskeleton and overall cell shape and morphology, which are critical components of cell polarity.\(^{17}\) The role of Rac1 in cell polarity has been shown by orientation of hair cells in the cochlea, convergent extension cellular movements and anterior-posterior body axis specification during development.\(^{18-20}\) We have recently demonstrated Rac1 to be a critical regulator of cardiomyocyte polarity and cardiac septation during heart development.\(^{21}\) However, the role of Rac1 in OFT development is unknown. The SHF progenitors in the dorsal splanchnic mesoderm can be further subdivided into two subdomains: the anterior and the posterior SHF.\(^{22,23}\) A *Mef2c-Cre* transgenic mouse has been developed that drives expression of Cre recombinase solely in the anterior subdomain of the SHF, which contributes to the right ventricle and the OFT. Specifically, the Cre recombinase sequence in the *Mef2c-Cre* transgenic mouse is under regulatory control of an anterior heart field specific enhancer and promoter, which was first discovered by Dodou et al.\(^{8,24}\) In order to study the role of Rac1 in OFT formation, we generated an anterior SHF-specific deletion of *Rac1* using the *Mef2c-Cre* transgenic mouse as described previously.\(^{21}\) We show that Rac1 signaling in the anterior SHF is
critical for progenitor cellular organization in the splanchnic mesoderm and disruptions in
Rac1 signaling result in a spectrum of OFT defects, along with aortic valve defects. Furthermore, we showed that a Rac1 deficiency, restricted to the anterior SHF, leads to neural crest cell migration defects.

3.3 Methods

3.3.1 Mice

*Rac1* \(^{f/f}\) (Stock #5550) and *mT/mG* C57BL/6 mice (Stock #7676) were purchased from Jackson Laboratory, Bar Harbor, Maine.\(^24, 25\) The anterior SHF specific *Mef2c-Cre* transgenic mice were rederived from embryos obtained from the Mutant Mouse Regional Resource Centers (MMRRC, Chapel Hill, North Carolina).\(^8\) A breeding program to generate C57BL/6 *Mef2c-Cre;Rac1* \(^{f/f}\) (Rac1 \(^{SHF}\)) mice was carried out and genotyping was performed as described previously.\(^21\) All mouse experiments and procedures were approved by the Animal Use Subcommittee at the University of Western Ontario in accordance with the guidelines of the Canadian Council of Animal Care.

3.3.2 Fate Mapping Analysis

Fate mapping analysis was performed using *Mef2c-Cre* and the *mT/mG* global double-fluorescent Cre reporter mice to trace the anterior SHF and derivatives.\(^8, 25\) A breeding strategy was carried out to generate *Mef2c-Cre;Rac1* \(^{f/f}\);*mT/mG* (Rac1 \(^{SHF};mT/mG\)) mice. The *mT/mG* mice possess *loxP* sites on either side of the membrane-targeted Tomato (mT) cassette with a membrane-targeted green fluorescent protein (GFP) cassette downstream. Before *Mef2c-Cre* mediated excision, mT (red fluorescence) is expressed in all cells. After *Mef2c-Cre* mediated excision, the mT
cassette is cleaved out and membrane-targeted GFP (mG) is expressed in all SHF progenitors and SHF-derived tissues.

3.3.3 Histological Analysis

Neonatal and embryonic samples were fixed overnight in 4% paraformaldehyde at 4°C, dehydrated and embedded in paraffin. Embryos were serially sectioned at 5 µm thick sections with a Leica RM2255 microtome and sections were mounted onto positively charged albumin/glycerin coated slides as previously described. Slides underwent a dewaxing process, stained with hematoxylin and eosin (H/E) or picrosirius red for morphological analysis. Images were captured with an Observer D1 light microscope (Zeiss, Germany).

3.3.4 Immunohistochemistry

For immunohistochemical staining on paraffin sections, samples underwent a deparaffin process and antigen retrieval was performed in sodium citrate buffer as described previously. The primary antibodies used for immunostaining include phosphohistone-H3 (phospho S10, Abcam), GFP (Abcam), α-actinin (Sigma), Sema3c (Santa Cruz) and AP2α (Santa Cruz). Slides were then incubated with biotinylated secondary antibody followed by incubation with avidin and biotinylated HPR from the Santa Cruz ImmunoCruz ABC staining system kit. Antigen was visualized with diaminobenzidine (DAB) substrate solution and slides were counterstained with hematoxylin. All images were captured with Zeiss Observer D1 microscope using AxioVision Rel 4.7 software. For frozen sections, embryos were fixed for one hour in 4% paraformaldehyde, cryoprotected in 30% sucrose and embedded in FSC22 frozen section media (Leica). Embryos were sectioned in a sagittal orientation at 10 µm thick and immunostained with
the primary antibodies for Scrib (Santa Cruz), aPKCζ (Santa Cruz), and active (non-phosphorylated) β-catenin (Cell Signaling). Additional staining was also performed with AlexaFluor 488 phalloidin (Life Technologies), AlexaFluor 647 wheat germ agglutinin (Invitrogen) and Hoechst 33342 (Invitrogen). A Zeiss LSM 510 Duo microscope with ZEN 2012 software (Zeiss, Germany) located at the Biotron Experimental Climate Change Research Center at Western University, London, Ontario was used to obtain confocal images.

3.3.5 Intrauterine Echocardiography

Echocardiography was performed on pregnant mice using Vevo 2100 (VisualSonics, Canada).26 Briefly, pregnant mice were anaesthetized with isoflurane and fur was shaved from the abdomen to better image embryos. A map was drawn of the location of embryos relative to each other to facilitate harvesting of embryos after intrauterine echocardiography. A dynamically focused 40 MHz probe was used to obtain 2-dimensional images of embryonic hearts in short-axis view. Diastolic and systolic left ventricular internal diameters (LVID) were measured from M-mode recordings. Calculations were performed as followed: ejection fraction (EF, %) = [(LVIDd)³ - (LVIDs)³]/(LVIDd)³×100. Fractional shortening (FS, %) = (LVIDd-LVIDs)/LVIDd×100. To assess aortic valve regurgitation, the transducer was aligned with the long axis of the heart and Color flow and Pulsed-Wave Doppler were used to visualize direction of blood flow in the aorta. In Color Doppler mode, red indicated flow towards the transducer and blue indicated blood flow away from the transducer. Pulsed-Wave Doppler mode was used to measure direction of blood flow, bloodflow velocity and duration of the aortic valve regurgitation.27
3.3.6 Statistical Analysis

Data are presented as means ± SEM. When data passed a normality test, an unpaired Student’s *t* test was employed to test for significance. Nonparametric Mann-Whitney test was used when samples size was 3 per group. Differences were considered significant at *P*<0.05.

3.4 Results

3.4.1 Early defects in the splanchnic mesoderm of *Rac1*<sup>SHF</sup>

Lineage tracing analysis using *Mef2c-Cre* and *mT/mG* global double fluorescent reporter mice revealed that lack of Rac1 in the anterior SHF resulted in defects in splanchnic mesoderm at E9.5. Large acellular spaces were observed between GFP<sup>+</sup> SHF progenitor cells and less cells resided in the overall splanchnic mesoderm region of E9.5 *Rac1*<sup>SHF</sup>;*mTmG* embryos (Fig. 3.1C, D) compared to littermate *Mef2c-Cre;mTmG;Rac1<sup>f/+</sup>* controls (Fig. 3.1A, B). The number of GFP<sup>+</sup> SHF progenitor cells in the splanchnic mesoderm was significantly less in *Rac1*<sup>SHF</sup> embryos compared to controls (Fig. 3.1E). Before differentiation, the SHF progenitor population in the splanchnic mesoderm is highly proliferative. To analyze SHF proliferation, phosphohistone-H3 (pHH3) immunostaining was performed on sagittal sections of E9.5 embryos (Fig. 3.1F, G). The proliferation rate of SHF progenitors in E9.5 *Rac1*<sup>SHF</sup> was significantly reduced compared to littermate *Rac1<sup>f/+</sup>* controls (Fig. 3.1H). This reduced proliferation rate likely resulted in a reduced number of SHF progenitors within the *Rac1*<sup>SHF</sup> splanchnic mesoderm. Overall, these results define a requirement for Rac1 signaling in SHF progenitor cell proliferation.
Figure 3.1. Early defects in E9.5 Rac1<sup>SHF</sup> splanchnic mesoderm.

Lineage tracing with *Mef2c-Cre;mTmG* showed a decreased number of GFP<sup>+</sup> SHF cells along with large acellular spaces in the splanchnic mesoderm of *Rac1<sup>SHF</sup>;mT/mG* (C, D) compared to littermate control embryos (A, B). The number of GFP<sup>+</sup> SHF progenitor cells in the splanchnic mesoderm was significantly reduced in E9.5 *Rac1<sup>SHF</sup>;mT/mG*
compared to controls (E). Immunostaining for phospho-histone H3 (pHH3) in the splanchnic mesoderm (F, G) showed a reduced proliferation rate in Rac1<sup>SHF</sup> SHF progenitors compared to Rac1<sup>f/f</sup> controls (H). *P<0.05, **P<0.01 by Student’s t-test, n=4-5 embryos per group. Scale bars: 100 µm (A, C), 10 µm (B, D), 20 µm (F, G). OFT, outflow tract; IFT, inflow tract; SpM, splanchnic mesoderm.

3.4.2 Disruption of cell shape and organization in Rac1<sup>SHF</sup> splanchnic mesoderm

SHF progenitor cells possess apicobasal polarity which form a polarized epithelial layer in the dorsal pericardial wall.<sup>10</sup> Our data show that cellular organization was severely disrupted in the E9.5 Rac1<sup>SHF</sup> anterior SHF progenitors (Fig. 3.2). The basolateral and apical domains of the Rac1<sup>f/f</sup> splanchnic mesoderm were marked by Scribble and aPKCζ, respectively. In addition, the SHF progenitors have a cuboidal shape, with neighbouring cells aligned with one another to form the epithelial layer (Fig. 3.2A-C). In comparison, organization of the Rac1<sup>SHF</sup> SHF epithelial layer was lost and SHF progenitor cells exhibited a rounded morphology. Cellular organization in the E9.5 Rac1<sup>SHF</sup> splanchnic mesoderm was disturbed where the basal domains of neighbouring SHF progenitors were no longer aligned with one another and the cells displayed a rounded morphology (Fig. 3.2D-F). Orientation of the long axis of the anterior SHF progenitor cells were measured relative to the axis of the dorsal pericardial wall (Fig. 3.2G) and it was found that E9.5 Rac1<sup>SHF</sup> SHF progenitor cells had a random, unorganized orientation compared to Rac1<sup>f/f</sup> controls. Rac1<sup>f/f</sup> SHF progenitors had an orientation range of 62 – 89.5°, averaging 78.5° relative to the dorsal pericardial wall.
axis. In comparison, \(Rac1^{SHF}\) SHF progenitors oriented with a significantly smaller angle averaging 41.8° relative to the dorsal pericardial wall axis (Fig. 3.2H). Cell-cell junctions, as marked by active (non-phosphorylated) \(\beta\)-catenin expression, was disrupted in the E9.5 \(Rac1^{SHF}\) splanchnic mesoderm compared to \(Rac1^{ff}\) controls, further supporting a disruption in SHF progenitor cell organization (Fig. 3.2I, J). Taken together, these findings support a critical role of Rac1 signaling in regulation of overall SHF progenitor cell shape and maintenance of cell organization.
Figure 3.2. Disrupted apicobasal cell polarity and orientation in Rac1SHF splanchnic mesoderm.

In E9.5 Rac1f/f SHF progenitors, the basolateral domain is marked by Scribble (B, arrows) and the apical domain is marked by aPKCζ (A, arrowheads). The SHF progenitors have a distinct cuboidal shape, forming an organized epithelial layer (C). Polarity is disrupted in Rac1SHF SHF progenitors where the Scribble+ basal domains (E, arrows) and aPKCζ+ apical domains (D, arrowheads) of individual cells are no longer aligned with neighbouring cells due to the rounded morphology of the SHF progenitors (F). C is an overlay of A and B. F is an overlay of D and E. The angle of E9.5 SHF progenitor cell long axis (dashed line) was measured relative to the axis of the dorsal pericardial wall (dashed arrow line) to obtain the degree of orientation (G). Images in G are from C and F with an overlay of schematic axis and angle measurements. E9.5 Rac1f/f SHF progenitor cells were approximately perpendicular to the dorsal pericardial wall axis. E9.5 Rac1SHF SHF progenitors had a random range of orientation with an average of 41.8° (H). ***P<0.001 by Student’s t-test, n=4 each group. Active (non-phosphorylated) β-catenin marked cell-cell junctions in E9.5 Rac1f/f SHF progenitors (I, arrows). In comparison, cell-cell junctions were disrupted in E9.5 Rac1SHF SHF progenitors (J). Scale bars: 10 µm (A-F, I-J).

3.4.3 Loss of Rac1 in the SHF results in shortened OFT and myocardial defects of the OFT

Sufficient lengthening of the heart tube from SHF progenitors ensures convergence of the inflow and outflow poles during cardiac looping. A shortened outflow
tract alters looping and leads to arterial pole misalignment defects such as double outlet right ventricle (DORV) and overriding aorta.\textsuperscript{29, 30} The sagittal length of the OFT was measured at E10.5 (Fig. 3.3A, B) and it was found that the $Rac^{SHF}$ OFT was significantly shorter compared to controls (Fig. 3.3C). Closer analysis of the SHF-derived OFT myocardium revealed a disorganized myocardial layer in E10.5 $Rac^{SHF}$ OFT compared to controls (Fig. 3.3D-G). Myocardial cells in the $Rac^{f/f}$ OFT were aligned with one another and had a general oval or cuboidal cell shape (Fig. 3.3D, F). In contrast, myocardial cells in the $Rac^{SHF}$ OFT were not as well aligned and the shapes of the cells were not as distinctly oval or cuboidal (Fig. 3.3E, G). In addition, cell-cell adhesion junction marked by $\beta$-catenin was generally disrupted in the myocardium of E10.5 $Rac^{SHF}$ OFT compared to $Rac^{f/f}$ controls (Fig. 3.3H, I). Myocardial cells of $Rac^{f/f}$ OFT had distinct cell-cell borders with neighbouring myocardial cells and $\beta$-catenin was localized to the basolateral region. Basolateral localization of $\beta$-catenin was disrupted in the myocardium of E10.5 $Rac^{SHF}$ OFT. These results suggest an important role of Rac1 in generating the cellular organization of the myocardial layer and lengthening of the OFT.
Figure 3.3. Defects in early $Rac1^{SHF}$ outflow tract development.

(A-C) Length of the OFT at E10.5 was measured in sagittal sections (A, B) and was found to be significantly shorter in $Rac1^{SHF}$ compared to $Rac1^{F/F}$ controls (C). *$P<0.05$ by Student’s t-test, n=4-5 each group. (D, E) Wheat germ agglutinin (WGA) staining to mark cell membranes of SHF cells in the OFT at E10.5 show a disorganized myocardial layer in $Rac1^{SHF}$ compared to $Rac1^{F/F}$ littermate controls. F and G are schematic diagrams of D and E, respectively. (H, I) Active (non phosphorylated) β-catenin staining is reduced
at cell-cell junctions in E10.5 Rac1<sup>SHF</sup> OFT myocardial cells. White arrows indicate cell-cell adhesion sites. Dotted white lines indicate boundaries of the OFT myocardial layer in D, E and H, I. Scale bars: 100 μm (A, B), 10 μm (D, E, H, I). Myo indicates myocardium.

### 3.4.4 OFT defects in Rac1<sup>SHF</sup> hearts

Examination of Rac1<sup>SHF</sup> hearts revealed a spectrum of OFT defects from E14.5 to P0 that was 100% penetrant (Table 3.1). The majority of the defects found were OFT alignment defects. Over one third of Rac1<sup>SHF</sup> hearts (23/64) displayed DORV (Fig. 3.4A-D) and over another one third (24/64) exhibited an overriding aorta (Fig. 3.4E, F). Transposition of the great arteries (TGA) was found in one P0 Rac1<sup>SHF</sup> sample (Fig. 3.4G, H). In addition to defects in OFT alignment, defects in septation of the OFT resulting in persistent truncus arteriosis (PTA) or common arterial trunk was observed in 9.4% (6/64) of Rac1<sup>SHF</sup> hearts (Fig. 3.4I, J). Narrowing of the great arteries was also observed in Rac1<sup>SHF</sup> hearts including stenosis of the pulmonary artery (Fig. 3.4K, L) and aortic atresia (Fig. 3.4M, N). In one case of aortic atresia in Rac1<sup>SHF</sup> hearts, aortic valves were also severely malformed (Fig. 3.4O, P). In addition, we observed abnormalities in aortic arch artery remodeling resulting in a retroesophageal right subclavian artery or a vascular ring, a congenital defect in which vascular structures surround and constrict the esophagus and trachea. This was evident in 18.8% (12/64) of Rac1<sup>SHF</sup> hearts and of the 12 samples that had a vascular ring, 3/12 had a right-sided aortic arch (Fig. 3.5A-F and Supplemental Videos and Footnotes). Taken together, all of these data strongly support a critical requirement for Rac1 signaling in anterior SHF progenitors for normal OFT morphogenesis.
Figure 3.4. Spectrum of OFT defects found in E14.5 - P0 Rac1\textsuperscript{SHF} hearts.

Double outlet right ventricle (DORV) was found in E15.5 Rac1\textsuperscript{SHF} hearts (A-D). The pulmonary artery (PA) connects to the RV in both Rac1\textsuperscript{SHF} and Rac1\textsuperscript{ff} hearts (A, B). The Rac1\textsuperscript{SHF} aorta (arrow) incorrectly connects to the RV compared to Rac1\textsuperscript{ff} controls, where the aorta connects to the LV (C, D). Overriding aorta, where the aorta is positioned directly over a ventricular septal defect (arrow), was observed in Rac1\textsuperscript{SHF} hearts (E, F). Transposition of the great arteries (TGA) was found in one Rac1\textsuperscript{SHF} sample where the aorta and pulmonary artery openings were switched, which resulted in the aorta (Ao) connecting to the RV in Rac1\textsuperscript{SHF} and the pulmonary artery to the LV (G, H), respectively. Persistent truncus arteriosus (PTA), where the common OFT is not divided
into aorta and pulmonary, was observed in $Rac^I_{SHF}$ hearts (I, J). Stenosis of the pulmonary artery was observed in $Rac^I_{SHF}$ hearts (K, L). Aortic atresia was observed in $Rac^I_{SHF}$ hearts (M, N) and in one sample with aortic atresia, distinct aortic valves were absent (O, P). RA, right atrium; LA, left atrium. Scale bars: 100 µm.

**Figure 3.5. Vascular rings in E15.5 $Rac^I_{SHF}$ hearts.**

A right-sided aortic arch (AA) was observed in $Rac^I_{SHF}$ (D) hearts compared to $Rac^I_{f/f}$ controls (A). An aberrant retroesophageal right subclavian artery (SCA), which arose from aortic arch, was observed next to the trachea (Tr) and esophagus (Eo) in $Rac^I_{SHF}$ hearts (E) compared to $Rac^I_{f/f}$ controls (B). The SCA in $Rac^I_{SHF}$ hearts joined with the pulmonary artery (PA), forming a vascular ring (F) in $Rac^I_{SHF}$ hearts compared to controls (C). DA indicates dorsal aorta. Scale bars: 100 µm.
Table 3.1. Outflow tract defects in \( \text{Rac1}^{\text{SHF}} \) hearts (E14.5 – P0).

<table>
<thead>
<tr>
<th></th>
<th>TGA</th>
<th>PTA</th>
<th>DORV</th>
<th>Overriding aorta</th>
<th>Aortic atresia</th>
<th>Vascular ring</th>
</tr>
</thead>
<tbody>
<tr>
<td>N=64</td>
<td>1</td>
<td>6</td>
<td>23</td>
<td>32</td>
<td>5</td>
<td>12</td>
</tr>
<tr>
<td>%</td>
<td>1.6</td>
<td>9.4</td>
<td>35.9</td>
<td>50</td>
<td>7.8</td>
<td>18.8</td>
</tr>
</tbody>
</table>

TGA, transposition of great arteries; PTA, persistent truncus arteriosus; DORV, double outlet right ventricle. All 64 \( \text{Rac1}^{\text{SHF}} \) hearts had one or more OFT defects. No OFT defects were found in \( \text{Rac1}^{\text{f/f}} \) hearts (N=36).

3.4.5 Myocardialization defects in \( \text{Rac1}^{\text{SHF}} \) hearts

Myocardialization is a process where myocardial cells migrate and grow into the flanking mesenchyme. At E12, myocardial cells of the OFT begin to invade the neighbouring cushion mesenchyme. This leads to a gradual replacement of the mesenchymal tissue with myocardium to form the muscular outlet septum by E17.\(^{31}\) Abnormalities in muscularization of the OFT cushions was observed in E12.5 \( \text{Rac1}^{\text{SHF}} \) hearts. The \( \text{Rac1}^{\text{SHF}} \) OFT myocardium lacked a polarized morphology and did not extend as far into the cushion mesenchyme compared to controls (Fig. 3.6A, B). Muscularization of the proximal OFT septum is complete in P0 \( \text{Rac1}^{\text{f/f}} \) control hearts as shown by \( \alpha \)-actinin positive muscle tissue separating the aorta from the RV (Fig. 3.6C). In P0 \( \text{Rac1}^{\text{SHF}} \) hearts, the proximal OFT septum remained non-muscularized and remnant collagen rich mesenchymal tissue remained (Fig. 3.6D), staining positive for picrosirius red (Fig. 3.6E, F). \( \text{Rac1}^{\text{SHF}} \) samples with defects in muscularization of the proximal OFT septum also presented with OFT misalignment defects including overriding aorta and
DORVs. These results further support a crucial role for Rac1 signaling in regulating polarity, along with elongation and migration of cardiomyocytes during the process of OFT myocardialization.
Figure 3.6. Abnormalities in $Rac1^{SHF}$ OFT myocardialization.

E12.5 $Rac1^{SHF}$ OFT cardiomyocytes, marked by $\alpha$-actinin immunostaining (B), exhibited a blunted morphology instead of a polarized morphology and did not extend as far into the OFT cushions compared to controls (A). Red arrows indicate invading cardiomyocytes. The proximal OFT septum remained non-muscularized in P0 $Rac1^{SHF}$ hearts (D), indicated by an absence of $\alpha$-actinin staining (arrows in D) compared to control (C) where the septum is muscularized (arrow in C). The non-muscularized proximal OFT septum in $Rac1^{SHF}$ hearts (F) stained positive for picrosirius red (arrows in F), indicating that this tissue remained mesenchymal compared to controls (E) which had become muscularized (arrow in E). N=3 for each staining per group. Ao, aorta; RV, right ventricle. Scale bars: 10 $\mu$m (A, B), 50 $\mu$m (C-F).

3.4.6 Decreased cardiac neural crest cell migration in $Rac1^{SHF}$

The aortic arch artery and OFT septation defects observed in $Rac1^{SHF}$ hearts suggests abnormal formation or remodeling of the arteries involving cardiac neural crest cells. Semaphorin 3c (Sema3c) is a secreted glycoprotein part of the semaphorin protein family, which plays a role in axon guidance. Sema3c is expressed in the OFT myocardium and acts as a chemoattractant, navigating neural crest cells to the OFT. Neural crest cells express the multimeric complexes of plexins and neuropilin, which are the receptors that recognize semaphorin ligands. Interactions between SHF progenitors and cardiac neural crest cells have been shown to be critical for development of the aortic arches and semilunar valves. Sema3c expression was analyzed in $Rac1^{SHF}$ hearts and intensity of Sema3c protein staining in E11.5 $Rac1^{SHF}$ OFT myocardium was found to be decreased compared to $Rac1^{f/f}$ controls (Fig. 3.7C, D, F). Along with this, the mRNA
level of Sema3c, as measured by real-time PCR, was significantly reduced in \( \text{Rac}^{1\text{SHF}} \) hearts compared to littermate controls at E13.5 (Fig. 3.7G). Consequently, this would be predicted to result in a reduction in the chemoattractant signal attracting the migrating neural crest cells into the developing heart. The number of migrating cardiac neural crest cells, marked by the transcription factor AP2α, was significantly decreased in the region between the foregut and pericardial cavity in E10.5 \( \text{Rac}^{1\text{SHF}} \) embryos (Fig. 3.7A, B, E). Overall, these results suggest that disruption of Rac1 signaling in the SHF-derived OFT myocardium leads to reduced expression of axonal guidance signals that attract migrating neural crest cells into the developing OFT.
Figure 3.7. Defects in neural crest cell contribution to OFT development in $Rac1^{SHF}$ hearts.

(A, B) AP2α immunostaining was performed to mark neural crest cells in the pharyngeal region of E10.5 samples. (C, D) Sema3c immunostaining (brown color) in $Rac1^{SHF}$ and $Rac1^{ff}$ OFT myocardium at E11.5. (E) The number of neural crest cells was significantly reduced in E10.5 $Rac1^{SHF}$ pharyngeal arches compared to controls. (F) Intensity of Sema3c staining in the E11.5 OFT myocardium was ranked on a scale from 1 to 5.
Overall intensity of Sema3c staining was reduced in \( \text{Rac1}^{\text{SHF}} \) compared to \( \text{Rac1}^{\text{f/f}} \) controls. (G) The level of \text{Sema3c} mRNA in \( \text{Rac1}^{\text{SHF}} \) hearts at E13.5 was significantly reduced compared to controls. \(*P<0.05\) by unpaired Student’s t-test, n=4-5 hearts (E, F) and 7-8 hearts (G) per group. FG, foregut; PC, pericardial cavity. Scale bars: 20 µm (A, B), 10 µm (C, D).

3.4.7 Aortic valve defects in \( \text{Rac1}^{\text{SHF}} \) hearts

Formation of valves involves a complex process of cushion formation, elongation, valve remodelling and maturation. Both SHF progenitors and cardiac neural crest cells contribute to aortic valve development. Neural crest cells are known to be involved in late gestation remodelling and maturation of the aortic valves. Thus, we predicted that the decreased number of migrated cardiac neural crest cells into \( \text{Rac1}^{\text{SHF}} \) hearts would also affect development of \( \text{Rac1}^{\text{SHF}} \) aortic valves. P0 \( \text{Rac1}^{\text{SHF}} \) heart sections were analyzed and found to have large, thickened aortic valve leaflets compared to controls which in contrast had undergone remodeling and matured into thin, elongated leaflets (Fig. 3.8A, B). Cell density of the aortic valve leaflets was also significantly decreased in P0 \( \text{Rac1}^{\text{SHF}} \) hearts compared to P0 \( \text{Rac1}^{\text{f/f}} \) hearts (14.3 ± 1.0 vs. 17.8 ± 0.8 cells/1000 µm², \(*P<0.05\), n=5-7), suggesting defects in ECM remodeling, which is necessary for the dense packing of cells in the elongating valves.

To assess glycosaminoglycans (GAG), a component of ECM in the aortic valve, toluidine blue staining was performed. The acellular space of the valve leaflets stained light purple indicating the presence of GAG (Fig. 3.8C, D). Notably, a significantly larger amount of GAG was present in \( \text{Rac1}^{\text{SHF}} \) aortic valves compared to littermate \( \text{Rac1}^{\text{f/f}} \) controls at P0 (\(**P<0.01\), Fig. 3.8G).
During early stages of development there is little to no collagen present in the semilunar valves. As the valves mature, collagen content increases, especially at the commissure of the valves.\textsuperscript{36} Analysis of P0 Rac1\textsuperscript{f/f} aortic valves after Masson’s trichrome staining revealed early signs of maturation with collagen present at the valve commissure (Fig. 3.8E). In comparison, P0 Rac1\textsuperscript{SHF} aortic valves were less mature and had little to no collagen present at the valve commissure (Fig. 3.8F).

Lineage tracing with mT/mG at E12.5 revealed decreased SHF progenitor contribution to the OFT cushions in Rac1\textsuperscript{SHF} hearts compared to control hearts which had numerous SHF-derived GFP\textsuperscript{+} cells in the cushion mesenchyme (Fig. 3.9A, B, black arrows). At E14.5, SHF contribution to the aortic valve leaflets continues to be severely reduced in Rac1\textsuperscript{SHF} hearts (Fig. 3.9C, D, black arrows). In addition, at both E12.5 and E14.5, contribution of SHF progenitors to the Rac1\textsuperscript{SHF} OFT myocardium seems to be decreased compared to controls (Fig. 3.9A-D, red arrows). This lineage tracing data strongly suggests an overall impairment in overall SHF progenitor cell contribution to the developing heart in Rac1\textsuperscript{SHF} embryos and a critical role for SHF Rac1 in maturation and remodeling of the aortic valves.
Figure 3.8. Aortic valve defects in Rac1^{SHF} hearts.

Over 30% (9/28) of P0 Rac1^{SHF} hearts exhibited thick aortic valve leaflets (B) compared to the thin, remodeled valves of controls (A). Toluidine blue staining showed glycosaminoglycans (GAG, light purple color) occupies the acellular space of Rac1^{SHF} and litttermate valve leaflets (C and D). Masson’s trichrome staining in Rac1^{ff} aortic valves showed collagen in the commissure of valve leaflets (E, arrows), which was absent in Rac1^{SHF} aortic valves (F, arrows). GAG positive area (light purple color) in each valve leaflet (C and D) was quantified in G. **P<0.01 by unpaired Student’s t-test, n=5-6 hearts per group. RCC, LCC and NCC are right, left and non-coronary cusps, respectively. Scale bars: 100 μm (A-D), 10 μm (E, F).

Figure 3.9. Decreased SHF contribution to Rac1^{SHF} OFT.

Fate mapping with mT/mG reporter shows a decreased SHF progenitor contribution to the OFT myocardium (red arrows in B and D) in E12.5 and E14.5 Rac1^{SHF} hearts compared
to controls (red arrows in A and C). SHF contribution to the valve leaflets was also severely reduced in Rac1<sup>SHF</sup> hearts compared to controls (black arrows, A-D). Paraffin sections were immunostained with anti-GFP. Scale bars: 20 µm.

### 3.4.8 Abnormal aortic valve function in Rac1<sup>SHF</sup> hearts

The observed aortic valve defects would be predicted to result in functional impairment of the valves. To assess the function of the malformed Rac1<sup>SHF</sup> aortic valves, intrauterine echocardiography at E18.5 was performed. Embryos from timed pregnant females were analyzed prior to genotyping and thus echo analysis was performed in a blinded manner. All E18.5 Rac1<sup>f/f</sup> control embryos did not demonstrate any evidence of aortic valve regurgitation (Fig. 3.10A). On the other hand, E18.5 Rac1<sup>SHF</sup> embryos were found to have severe aortic regurgitation during diastole (Fig. 3.10B). In addition, left ventricular internal diameter during systole (LVIDs) was significantly increased in Rac1<sup>SHF</sup> hearts while no significant differences were observed in left ventricular internal diameter during diastole (LVIDd). Both ejection fraction and fractional shortening in E18.5 Rac1<sup>SHF</sup> embryos were also significantly reduced compared to controls (Table 3.2). Decreased left ventricular function in Rac1<sup>SHF</sup> hearts is likely related to the defects in myocardial development reported in our previous study. Overall, this echocardiography data further defines a requirement for SHF Rac1 in development and functional maturation of the aortic valves.
Figure 3.10. Aortic valve regurgitation in $Rac1^{SHF}$ hearts.

Intrauterine pulsed-wave echocardiography at E18.5 showed that $Rac1^{SHF}$ hearts had severe aortic regurgitation during diastole (B, red arrows), which is not observed in $Rac1^{f/f}$ littersmates (A). White arrows in A and B indicate forward blood flow during systole. Shown are representatives of 5 fetuses per group from 4 mothers.
Table 3.2. Intrauterine echocardiography in E18.5 \(Rac1^{SHF}\) hearts.

<table>
<thead>
<tr>
<th></th>
<th>Rac1(^{f/f}) ((n=5))</th>
<th>Rac1(^{SHF}) ((n=5))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duration of backflow (ms)</td>
<td>0</td>
<td>198 ± 20</td>
</tr>
<tr>
<td>Velocity of backflow (mm/s)</td>
<td>0</td>
<td>256 ± 96</td>
</tr>
<tr>
<td>LVIDd (mm)</td>
<td>0.87 ± 0.03</td>
<td>0.89 ± 0.05</td>
</tr>
<tr>
<td>LVIDs (mm)</td>
<td>0.57 ± 0.02</td>
<td>0.66 ± 0.03 *</td>
</tr>
<tr>
<td>Ejection Fraction (%)</td>
<td>33.88 ± 1.15</td>
<td>25.64 ± 1.42 **</td>
</tr>
<tr>
<td>Fractional Shortening (%)</td>
<td>70.99 ± 1.49</td>
<td>58.71 ± 2.37 **</td>
</tr>
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</table>

LVIDd, left ventricular internal diameter in diastole; LVIDs, left ventricular internal diameter in systole. *\(P<0.05\), **\(P<0.01\) by unpaired Student’s t-test.

3.5 Discussion

Recent studies have established cell and tissue polarity as important regulators of cardiac OFT development, especially factors involved in the PCP pathway.\(^ {11, 12}\) However, the role of Rac1 in cardiac OFT development is not clear. In the present study, we demonstrated that a deficiency of Rac1 signaling in the anterior SHF disrupted progenitor cell shape and overall epithelial organization in the splanchnic mesoderm. In addition, the proliferation rate of SHF progenitors was decreased with a loss of Rac1 signaling. Subsequently, as heart development progressed, these early defects in the SHF splanchnic mesoderm resulted in a spectrum of OFT and aortic valve defects. We also showed that Rac1 deficiency disrupted the levels of Sema3c, a chemoattractant for cardiac neural crest cells, generated by the SHF-derived OFT myocardium, that would provide a
mechanism for our observed reduction in migrating neural crest cells observed in the developing OFT (Fig. 3.11).

Figure 3.11. Schematic diagram of mid-sagittal section of E9.5 splanchnic mesoderm.

At E9.5, the $\text{Rac1}^{\text{ff}}$ anterior SHF progenitors in the splanchnic mesoderm are organized into a polarized epithelium (a). Chemotactic signals, such as Sema3c, secreted by the OFT myocardium act as axonal guidance cues for migration of cardiac neural crest (NC)
cells from the neural tube (A). In Rac1\textsuperscript{SHF} embryos, the anterior SHF progenitors are rounded and disorganized, displaying a loss of polarized epithelium characteristics (b). Expression of chemotactic signals is reduced, resulting in decreased migration of neural cells into the OFT (B).

Intricate signaling cascades exist between the cardiac neural crest and the SHF. Defects in the cardiac neural crests can affect the SHF progenitors and vice versa. Ablation of cardiac neural crest cells lead to a failure of the SHF progenitors to lengthen the heart tube and to contribute to the OFT myocardium\textsuperscript{30,36}. In addition to ablation, tissue-specific genetic deletions in the cardiac neural crest cells, which affect signaling pathways including Bmp, Smad and in the transcription factor Tbx3, all have secondary effects on the SHF and result in defects to OFT remodeling, alignment and elongation\textsuperscript{37-39}. Conversely, tissue-specific genetic deletions in the SHF tissue, including in Notch and Tbx1, can also have a non-cell autonomous effect on migration of cardiac neural crest cells into the developing heart\textsuperscript{7,40}. Our study showed that conditional deletion of Rac1 in the SHF reduced the migration of cardiac neural crest cells into the pharyngeal region, supporting a role for Rac1 in supporting the intercellular signaling that exists between the SHF and neural crest. The mechanism by which Rac1 signaling regulates expression of the neural crest chemoattractant Sema3c in the SHF-derived OFT myocardium remains to be determined. Studies have shown that Rac1 activates c-jun N-terminal kinase (JNK) to increase gene expression, including expression of the zinc-finger transcription factor Gata6\textsuperscript{41,42}. Sema3c can be transcriptionally regulated by Gata6 and in humans, Gata6 mutations can cause PTA through disruptions in the semaphorin-plexin signaling pathway\textsuperscript{43,44}. Whether this Rac1-JNK-Gata6-Semaphorin signaling pathway is conserved
in the SHF-derived OFT myocardium, and subsequently signals to the migrating cardiac neural crest during cardiac morphogenesis remains to be determined in future studies.

The OFT defects observed in the $Rac1^{SHF}$ embryos in the present study are likely due to a combination of abnormalities in several development events which are linked to initial establishment of cell polarity, including proliferation and migration. Firstly, proliferation of the $Rac1^{SHF}$ SHF progenitors in the splanchnic mesoderm is dramatically reduced, thus decreasing the overall number of anterior SHF cells contributing to OFT development. The two biological processes of cell polarity and proliferation are coordinated during development, where the polarized state of a progenitor affects its proliferation rate. This linkage has been clearly observed in development of the nervous system.$^{45, 46}$ In this study, we showed that a deficiency of Rac1 signaling in the anterior SHF progenitors resulted in a loss of cellular organization and is correlated with a decrease in proliferation rate, further supporting a link between these two developmental processes. Francou et al has also demonstrated a correlation with direct alteration of the epithelial properties of the anterior SHF progenitors, resulting in reduced progenitor proliferation rate and ectopic differentiation of the SHF progenitor cells.$^{10}$ However, the direct mechanistic link between proliferation and cell polarity in the SHF progenitors remains unclear. In neural progenitors, apically localized aPKC attenuates the activity of p27Xic1, which is an inhibitor of cyclin-dependent kinase-2 (Cdk2). This leads to increased Cdk2 activity and shortening of the G1 and S phases, thus increasing the rate of proliferation.$^{47}$ Whether a similar mechanism exists in anterior SHF progenitors remains to be determined. Furthermore, concomitant to a decrease in SHF progenitor cell proliferation, a decreased migratory ability of the $Rac1^{SHF}$ anterior SHF progenitors to
add to the arterial pole of the heart tube likely contributed to the observed OFT defects. Establishment of cell polarity is intrinsically linked to migratory ability. Both polarization and migration are events dependent on dynamic changes to the actin cytoskeleton, which is regulated by Rac1.\textsuperscript{48} Evidence of impaired migratory ability of the $Rac1^{SHF}$ SHF during heart development was recently demonstrated in our previous study.\textsuperscript{21} Migration defects were also observed in the present study based on $mT/mG$ lineage tracing, which showed reduced contribution of SHF cells to the aortic valves and OFT, and impaired migration of cardiomyocytes into the $Rac1^{SHF}$ proximal outlet septum during the myocardialization process. Overall, these observations support establishment of SHF progenitor epithelial organization as critical to the developmental process.

Valve remodeling and development is regulated by coordinated actions between matrix metalloproteinase (MMPs) and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) families of zinc metalloproteinases.\textsuperscript{49} Studies have shown a role for Rac1 in the regulation of ECM degradation along with MMP expression and activity.\textsuperscript{50,51} The thickened $Rac1^{SHF}$ aortic valve leaflets along with decreased valve cell density and increased deposition of glycosaminoglacans observed in this study strongly suggest defects in valvular ECM degradation and remodeling. However, whether Rac1 signaling in the SHF-derived cells residing in the aortic valve leaflets also regulate the ECM through MMPs remains to be determined in future studies. Along with this, blood flow-induced hemodynamics can influence valve morphogenetic cues.\textsuperscript{52} Our previous study reported atrial and ventricular septal defects along with abnormalities in development of the ventricles in $Rac1^{SHF}$ hearts.\textsuperscript{21} Whether these structural defects
altered the hemodynamic flow and subsequently affected remodeling of the $Rac^{SHF}$ aortic valves remains to be determined.

In summary, our study is the first to show that Rac1 signaling in the anterior SHF is critical to normal cell organization in the splanchnic mesoderm. Loss of Rac1 leads to disruptions in anterior SHF progenitor cellular organization early in development and impacts subsequent steps of heart development, including OFT and aortic valve morphogenesis, along with signaling to the neural crest cells. Our study suggests that perturbed Rac1 signaling in the anterior SHF could account for some of the OFT defects observed in humans.

3.6 Footnotes

Supplemental Video Legends

**Video S1.** 3D reconstruction of OFT in E15.5 $Rac^{1f/f}$ heart. In $Rac^{1f/f}$ hearts, the aortic arch (pink) is located on the left side, giving rise to the ascending and descending aorta (red). The dorsal aorta runs parallel to the esophagus (yellow) and trachea (green). Pulmonary artery is blue.

**Video S2.** 3D reconstruction of vascular ring in E15.5 $Rac^{1SHF}$ heart. In $Rac^{1SHF}$ hearts, the aortic arch (pink) is located incorrectly on the right side, giving rise to the ascending and descending aorta (red). The dorsal aorta wraps around the esophagus (yellow) and trachea (green), forming a vascular ring. Pulmonary artery is blue.
This work was funded by operating grants (to Q.F. and T.A.D.) from Canadian Institutes of Health Research (CIHR) and Heart & Stroke Foundation of Ontario (HSFO). C.L. was supported by a Natural Sciences and Engineering Research Council (NSERC) Scholarship. Q.F. is a HSFO Career Investigator. The authors would like to thank Murong Liu for her assistance in mouse breeding and genotyping.
3.7 References


Chapter 4

Myocardium-specific deletion of Rac1 causes ventricular myocardium and outflow tract defects

A version of this chapter is in preparation for submission.

Carmen Leung, Xiangru Lu, Chi-chung Hui, Qingping Feng

Department of Physiology and Pharmacology, Department of Medicine (Q.F.), Schulich School of Medicine and Dentistry, Collaborative Program in Developmental Biology (C.L.), Lawson Health Research Institute (Q.F.), The University of Western Ontario, London, Ontario, Canada N6A 5C1 and Program in Developmental and Stem Cell Biology, Hospital for Sick Children, Toronto, Ontario, Canada M5G 1X8 (C.H.)

“Myocardium-specific deletion of Rac1 causes ventricular myocardium and outflow tract defects”
Chapter 4

4.1 Chapter Summary

Left ventricular noncompaction (LVNC) is a cardiomyopathy that can lead to arrhythmias, embolic events and heart failure. Despite our current knowledge of cardiac development, the mechanisms underlying noncompaction of the ventricular myocardium are still poorly understood. The small GTPase Rac1 acts as a crucial regulator of numerous developmental events; however, its role in ventricular myocardium development is unclear. In the present study, we tested the hypothesis that Rac1 signaling is critical for development of the ventricular compact myocardium. Using the ventricular myocardium specific \( Nkx2.5\)-\( Cre \) transgenic mouse crossed to \( Rac1^{ff} \), mice with a ventricular myocardium specific deletion of Rac1 were generated (\( Rac1^{Nkx2.5} \)). Embryonic \( Rac1^{Nkx2.5} \) hearts at E13.5 - E18.5 were collected for histological analysis. Overall, \( Rac1^{Nkx2.5} \) hearts displayed bifurcation of the ventricles, along with defective trabeculation formation and a thin compact myocardium. \( Rac1^{Nkx2.5} \) hearts also exhibited ventricular septal defects (VSDs) and double outlet right ventricle (DORV) or overriding aorta. Cardiomyocytes in \( Rac1^{Nkx2.5} \) hearts were not polarized and expression of Scrib, a PCP protein, was reduced. In addition, the proliferation rate of E9.5 \( Rac1^{Nkx2.5} \) ventricular myocardium was reduced compared to controls. A Rac1 deficiency in the ventricular myocardium impairs cardiomyocyte polarization and ventricular myocardium organization, along with proliferative growth of the heart. A spectrum of CHDs arise in \( Rac1^{Nkx2.5} \) hearts, implicating Rac1 signaling in the ventricular myocardium as a crucial regulator of OFT alignment, along with trabecular and compact myocardium growth and development.
4.2 Introduction

Congenital heart defects (CHDs) are the most common human birth defect, affecting up to 5% of live births. The severity of these types of defects range from simple defects with no symptoms at birth, such as a small atrial septal defect (ASD), to complicated defects that are life-threatening and require intervention, such as Tetralogy of Fallot (TOF). One particular defect known as left ventricular noncompaction (LVNC) or “spongy myocardium” has no treatment at present, with the major therapy being heart failure medications and anticoagulation medications. LVNC is classified as a rare genetic cardiomyopathy, occurring in 0.01% to 0.27% of the population, characterized by arrest of normal myocardium development, leading to a thin compact myocardial layer and an extensive non-compacted trabecular network. Noncompaction of the ventricular myocardium can increase the risk of cardiac embolism, atrial fibrillation, ventricular arrhythmia and heart failure. Although this defect occurs mostly in the LV, RV noncompaction has also been reported in less than one-half of LVNC patients.

The ventricles grow and mature through a process of proliferation and differentiation to form trabeculation and compact myocardium. In the mouse, trabecular formation begins at approximately E9.5 where cardiomyocytes grow to form protrusions of muscular ridges. The trabeculae then undergo a process of remodeling/compaction where the bases of the trabeculation thicken and collapse into the myocardial wall. Proliferation of the compact myocardium is concomitant with remodeling. By E14.5, mature trabeculation is formed, along with a thick, compact myocardium. Numerous
signaling pathways have been implicated in development of the ventricular chambers including Notch, BMP, FGF, retinoic acid and PCP signaling. In addition, various mouse models of ventricular noncompaction defect have been generated but the ventricular defects between the mouse models are not consistent, reflecting the complex process of ventricular myocardium development. To date, the complete signaling mechanisms underlying ventricular myocardium development are still not completely understood.

The small GTPase, Rac1, acts as a crucial regulator of numerous developmental events including proliferation, cell cycle progression, cell survival, differentiation and regulation of cell shape, morphology and polarity. Specifically, the importance of Rac1 signaling in embryonic heart development has emerged in recent studies. Our work has demonstrated a critical role for Rac1 signaling in the anterior SHF and anterior-SHF derived structures including the RV, interventricular septum and the OFT. Rac1 regulates cardiomyocyte polarization in the RV and formation of the cardiac apex. However, the role of Rac1 in development of the LV myocardium is unclear. In order to study the role of Rac1 in the ventricular myocardium, we generated a ventricular myocardium specific deletion of Rac1 using the Nkx2.5-Cre transgenic mouse, which has Cre recombinase activity in both the RV and LV. We show that Rac1 signaling is crucial for ventricular myocardium development and downregulation of Rac1 leads to a spectrum of CHDs including thin compact myocardium and defective trabeculation development, similar to the features of LVNC. Rac1 deficient hearts also had a bifid cardiac apex and OFT alignment defects. Furthermore, we showed that a Rac1 deficiency in the ventricular
myocardium disrupts proliferation along with the organization and polarization of cardiomyocytes.

4.3 Methods

4.3.1 Mice

The \textit{Rac1}^{f/f} C57BL/6 mouse line (Stock #5550) and \textit{mT/mG} C57BL/6 mouse line (Stock #7676) were purchased from Jackson Laboratory, Bar Harbor, Maine\textsuperscript{22, 23}. The \textit{Nkx2.5-Cre} C57BL/6 transgenic mouse was a generous gift from Dr. Chi-Chung Hui at SickKids Hospital, University of Toronto, originally generated by McFadden et al.\textsuperscript{21} The \textit{mT/mG} mouse is a global double-fluorescent Cre reporter mouse. This reporter mouse expresses membrane-targeted Tomato (mT) before Cre-excision and membrane-targeted GFP (mG) after excision of mT.\textsuperscript{23} A breeding program to generate \textit{Nkx2.5-Cre;Rac1}^{f/f} (Rac1\textsuperscript{Nkx2.5}) and \textit{Nkx2.5-Cre;mT/mG} mice was carried out and genotyping was performed as described previously.\textsuperscript{19} Genotyping primer sequences are listed in \textbf{Table 4.1}. Mouse experiments and procedures were approved by the Animal Use Subcommittee at the University of Western Ontario in accordance with the guidelines of the Canadian Council of Animal Care.
Table 4.1. Genotyping PCR primer sequences.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
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<tbody>
<tr>
<td>Nkx2.5-Cre</td>
<td>tgccacgaccaagtgacagc</td>
<td>ccaggttacgatatagttcatg</td>
</tr>
<tr>
<td>Rac1&lt;sup&gt;f/f&lt;/sup&gt;</td>
<td>tccaatctgtgctgcccatc</td>
<td>gatgetctaggggtgagcc</td>
</tr>
<tr>
<td>mTmG</td>
<td>ctetgtgcctctgtgcttct</td>
<td>cgagccggatcacaagcaata</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mutant reverse:</td>
</tr>
<tr>
<td></td>
<td></td>
<td>tcaatgggccccggtgtcgtt</td>
</tr>
</tbody>
</table>

4.3.2 Histological Analysis

Embryonic samples (thoracic cavity) were fixed overnight in 4% paraformaldehyde at 4°C, dehydrated and paraffin embedded. Samples were serially sectioned at 5 µm from the top of the aortic arch to the apex of the heart with a Leica RM2255 microtome. Sections were mounted onto positively charged albumin/glycerin coated microslides. Slides were stained with hematoxylin and eosin (H/E) for histological analysis and images were captured using a light microscope (Observer D1, Zeiss, Germany).

4.3.3 Immunohistochemistry

Immunohistochemical staining was performed on paraffin heart sections. Antigen retrieval was carried out in sodium citrate buffer (pH 6.0) at 92 °C using a BP-111
laboratory microwave (Microwave Research & Applications, Carol Stream, Illinois). Immunostaining was performed with primary antibodies for GFP (Abcam) and Scrib (Santa Cruz) followed by incubation with biotinylated secondary antibody and avidin and biotinylated HPR (Santa Cruz). Diaminobenzidine (DAB) substrate solution was used to visualize the substrate and slides were counterstained with hematoxylin. Images were captured with Zeiss Observer D1 microscope using AxioVision Rel 4.7 software. For proliferation and apoptosis analysis, E9.5 heart samples were fixed in 4% paraformaldehyde for one hour, cryoprotected in 30% sucrose and embedded in FSC22 frozen section media (Leica). Samples were sectioned in a sagittal orientation with a Leica cryostat at 10 µm thick onto glass slides. Slides were incubated with phosphohistone-H3 (phospho S10) (Abcam), cleaved caspase-3 (Cell Signaling) primary antibody, Alexa Fluor 647 wheat germ agglutinin (Invitrogen), Alexa Fluor 488 phalloidin (Life Technologies) and counterstained with Hoechst 33342 (Invitrogen). Confocal images were obtained at the Biotron Research Centre, Western University with a Zeiss LSM 510 Duo microscope using ZEN 2012 software (Zeiss, Germany).

4.3.4 Quantitative Real Time RT-PCR

Total RNA was isolated from E12.5 ventricular myocardium using the RNeasy Mini Kit (QIAGEN). Reverse transcription reaction was performed, as described previously. Briefly, M-MLV Reverse Transcriptase (Invitrogen) and EvaGreen qPCR Mastermix (Applied Biological Materials Inc.) was used for real time thermal cycling. 28S rRNA was used as an internal control. The Eppendorf Mastercycler Realplex Real-Time PCR machine was used and samples were amplified for 35 cycles. The mRNA level of Rac1 in
relation to 28S rRNA was determined using a comparative C<sub>T</sub> method. Real time RT-PCR primer sequences are listed in Table 4.2.

Table 4.2. Real time RT-PCR primer sequences.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
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<tr>
<td>Rac1</td>
<td>aacctgcctgcctcagtt</td>
<td>ttgtccagctgtgtccata</td>
</tr>
<tr>
<td>28S</td>
<td>ttgaaatccgggggagag</td>
<td>acattgttcaacatgccag</td>
</tr>
</tbody>
</table>

4.3.5 Western Blot Analysis

Rac1 protein expression from E12.5 ventricular myocardium was measured by western blot analysis. Briefly, 25 µg of protein from isolated ventricular tissue was separated by 12% SDS-Page gel and transferred to nitrocellulose membranes. Blots were probed with antibodies against Rac1 (1:500, Santa Cruz) and α-actinin (1:5000, Sigma). Blots were then washed and probed with horseradish peroxidase conjugated secondary antibodies (1:2500, Bio-Rad) and detected using an ECL detection method. Densitometry was then performed to quantify the signal.

4.3.6 Statistical Analysis

Data are presented as means ± SEM. An unpaired Student’s t test was employed to test for significance when data passed a normality test. Differences were considered significant at P<0.05.
4.4 Results

4.4.1 Generation of a transgenic mouse with a Rac1 deficiency in the ventricular myocardium

*Nkx2.5-Cre* transgenic mice and *Rac1*\textsuperscript{ff} mice were crossed to generate *Nkx2.5-Cre;Rac1*\textsuperscript{ff} \((Rac1^{Nkx2.5})\) offspring. The Cre recombinase in the *Nkx2.5-Cre* mouse line is activated after E8.5 and initial specification of cardiac progenitors.\textsuperscript{21} To confirm a knockdown in *Rac1* mRNA expression, E12.5 *Rac1^{Nkx2.5} ventricles were isolated for RNA and real-time PCR was performed. *Rac1* mRNA expression was significantly decreased by approximately 35\% in E12.5 *Rac1^{Nkx2.5} ventricular myocardium compared to littermate *Rac1*\textsuperscript{ff} controls (Fig. 4.1A). Protein from E12.5 *Rac1^{Nkx2.5} ventricular myocardium was also isolated for western blot analysis. The ratio of Rac1 protein expression to \(\alpha\)-actinin was reduced by 42\% in E12.5 *Rac1^{Nkx2.5} ventricular myocardium compared to littermate *Rac1*\textsuperscript{ff} controls (Fig. 4.1B). These results confirm that *Nkx2.5-Cre* mediated recombination sufficiently downregulates *Rac1* expression in the ventricular myocardium of the developing embryonic heart.
Figure 4.1. Generation of mouse line with a ventricular myocardium specific deletion of Rac1 \( (Rac1^{Nkx2.5}) \).

\( Nkx2.5\)-Cre transgenic mice were crossed to \( Rac1^{f/f} \) to generate \( Rac1^{Nkx2.5} \) mice. Rac1 mRNA expression was significantly reduced in E12.5 \( Rac1^{Nkx2.5} \) ventricular myocardium compared to \( Rac1^{f/f} \) littermates (A). Western blot analysis of E12.5 ventricular myocardium showed a significant decrease in Rac1 protein in \( Rac1^{Nkx2.5} \) hearts compared to controls (B). *\( P<0.05 \) by Student’s t-test.

4.4.2 Lineage tracing of \( Nkx2.5\)-Cre transgenic mouse

To trace where the Cre recombinase is active in \( Nkx2.5\)-Cre transgenic hearts, \( Nkx2.5\)-Cre mice were crossed to \( mT/mG \) reporter mice, which marks all tissues possessing Cre recombinase activity with GFP. McFadden et al, the group who first created the \( Nkx2.5\)-Cre transgenic mouse, used a lacZ reporter to show that the Cre recombinase is active throughout the ventricular myocardium with minimal recombination in the OFT and atria. At E12.5, we showed with the \( Nkx2.5\)-Cre;\( mT/mG \) mouse that the Cre recombinase is active throughout the ventricular myocardium and a large portion of the atria (Fig. 4.2A). In addition, a majority of the pulmonary artery myocardium and only some cells in the aorta, were GFP\(^+\) in E12.5 \( Nkx2.5\)-Cre;\( mT/mG \) hearts (Fig. 4.2B). Closer analysis of the aortic valves showed that some of the cells in the early aortic valves were also GFP\(^+\) in the \( Nkx2.5\)-Cre;\( mT/mG \) hearts (Fig. 4.2C). Furthermore, closer analysis of the epicardium and endocardial cells at E18.5 showed that these cells remained RFP\(^+\), indicating no Cre recombinase activity in these cell types (Fig. 4.2D-F). Thus, the \( Nkx2.5\)-Cre mouse is an effective transgenic mouse to use to
study development of the ventricular myocardium, as the majority of these cells are GFP

Figure 4.2. Lineage tracing with Nkx2.5-Cre;mtmG mice.

GFP immunostaining of E12.5 Nkx2.5-Cre;mtmG paraffin heart sections showing Cre recombinase activity in the ventricular myocardium (A), OFT (B) and aortic valve leaflets (C). Cryosections of E18.5 Nkx2.5-Cre;mtmG hearts showed that the epicardium (arrows) and endothelial cells (arrowhead) remain RFP+ in Nkx2.5-Cre;mtmG hearts (D-F). RV, right ventricle; LV, left ventricle; PA, pulmonary artery; Ao, aorta; epi, epicardium. Scale bars: 250 μm (A-C), 10 μm (D-F).

4.4.3 Congenital heart defects in Rac1Nkk2.5 hearts

All P0 Rac1Nkk2.5 offspring were found dead at birth (n=5) from two litters. Gross morphological analysis of these P0 Rac1Nkk2.5 hearts revealed a bifid cardiac apex, similar
to what was observed and reported in our previous study with a *Mef2c-Cre* anterior second heart field-specific deletion of *Rac1*\(^{19}\). Examination of all *Rac1\(^{Nkx2.5}\)* hearts at earlier embryonic time points showed evidence of a bifid cardiac apex as well, along with enlarged atria (Fig. 4.3A). In addition, *Rac1\(^{Nkx2.5}\)* hearts had incomplete development of the interventricular septum, resulting in a ventricular septal defect (VSD) (Fig. 4.3B-C, Table 4.3). Alignment of the outflow tract (OFT) to the ventricles was also defective in *Rac1\(^{Nkx2.5}\)* hearts compared to littermate *Rac1\(^{f/f}\)* controls. A double outlet right ventricle (DORV) was observed in 11 of the 17 *Rac1\(^{Nkx2.5}\)* hearts (Fig. 4.3D-G, Table 4.3) and 6 of the 17 *Rac1\(^{Nkx2.5}\)* hearts exhibited an overriding aorta (Table 4.3). In addition, both the left ventricle (LV) and right ventricle (RV) of *Rac1\(^{Nkx2.5}\)* hearts had defective trabeculation (Fig. 4.4A-D). The compact myocardium of both *Rac1\(^{Nkx2.5}\)* ventricles at E15.5 was poorly formed and significantly thinner compared to littermate *Rac1\(^{f/f}\)* controls (Fig. 4.4E). These findings suggest a critical role for Rac1 in interventricular septum formation, OFT alignment and development of the trabecular and compact ventricular myocardium.

**Table 4.3. Congenital heart defects in *Rac1\(^{Nkx2.5}\)* hearts (E14.5 – P0).**

<table>
<thead>
<tr>
<th></th>
<th>Bifid apex</th>
<th>VSD</th>
<th>DORV</th>
<th>Overriding aorta</th>
<th>Thin myocardium</th>
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<tr>
<td>N=17</td>
<td>17</td>
<td>17</td>
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<td>%</td>
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<td>100</td>
<td>64.7</td>
<td>35.3</td>
<td>100</td>
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</table>
VSD, ventricular septal defect; DORV, double outlet right ventricle. All 17 $Rac1^{SHF}$ hearts had more than one type of CHD. No CHD defects were found in littermate E14.5-P0 $Rac1^{l/l}$ hearts (n=11).
Figure 4.3. Congenital heart defects in $Racl^{Nkx2.5}$.  

Bifid cardiac apex in an E16.5 $Racl^{Nkx2.5}$ heart. Arrow indicates bifurcation between the RV and LV (A). Ventricular septal defect (arrowhead) was found in E15.5 $Racl^{Nkx2.5}$ hearts. Arrow indicates bifid cardiac apex (B, C). Double outlet right ventricle (DORV) was found in E15.5 $Racl^{Nkx2.5}$ hearts. The pulmonary artery connects to the RV in both $Racl^{f/f}$ controls and $Racl^{Nkx2.5}$ hearts (D, E). The $Racl^{Nkx2.5}$ aorta (G) incorrectly connects to the RV compared to $Racl^{f/f}$ controls, where the aorta connects to the LV (F). RA, right atrium; LA, left atrium. Scale bars: 500 µm.
Figure E

Wall Thickness (µm)

- Rac1^{f/f} (n=6)
- Rac1^{Nkx2.5} (n=5)

RV

LV

***
Figure 4.4. Ventricular myocardium defects in Rac1<sup>Nkx2.5</sup> hearts.

The RV and LV myocardium and trabeculation of E15.5 Rac1<sup>Nkx2.5</sup> hearts (B, D) were severely defective compared to Rac1<sup>f/f</sup> controls (A, C). The thickness of the compact myocardial wall in E15.5 Rac1<sup>Nkx2.5</sup> RV and LV was significantly decreased compared to littermate controls (E). Double-headed arrows in A-D indicate measurements of wall thickness. ***P<0.001 by Student’s t-test. Scale bars: 100 µm.

4.4.4 Loss of cell polarity and organization in Rac1<sup>Nkx2.5</sup> hearts

To analyze the organization of the cardiomyocytes in the ventricular myocardium, E18.5 Rac1<sup>Nkx2.5</sup> heart sections were stained with wheat germ agglutinin (WGA), which marks cell borders, and phalloidin to mark F-actin filaments. WGA staining revealed rounded, spherically shaped cardiomyocytes in Rac1<sup>Nkx2.5</sup> hearts, in both the RV and LV (Fig. 4.5B). In comparison, littermate ventricular myocardium had cardiomyocytes that had undergone polarization and were an elongated shape in both the RV and LV (Fig. 4.5A). In addition, Rac1<sup>Nkx2.5</sup> hearts had severely disrupted F-actin filament organization (Fig. 4.5D) compared to controls, which had long, parallel running F-actin filaments throughout the myocardium (Fig. 4.5C). These findings are similar to the observations from our first study, where Rac1 was deleted in the SHF and all SHF-progenitors in Rac1<sup>SHF</sup> hearts. The RV in the SHF-Rac1 deficient hearts showed similar myocardial disorganization as the ventricular myocardium of Rac1<sup>Nkx2.5</sup> hearts in this study. This data suggests a crucial role for Rac1 in the organization, polarization and elongation of cardiomyocytes during embryonic heart development.
Figure 4.5. Disruption of cardiomyocyte polarity and organization in $Rac1^{Nkx2.5}$ hearts.

WGA staining in E18.5 cryosections show rounded cardiomyocytes in $Rac1^{Nkx2.5}$ ventricular myocardium (B) compared to the elongated cardiomyocytes in controls (A). F-actin filament organization is disrupted in $Rac1^{Nkx2.5}$ myocardium (D) compared to controls (C). Scale bars: 10 µm.

4.4.5 Decreased Scrib protein in $Rac1^{Nkx2.5}$ hearts

Scrib genetically interacts with Rac1 and Scrib protein forms a complex with the Rac1-GEF, β-PIX, and Rac1 in the developing myocardium. Loss of either Scrib or Rac1 leads to a reduction in membrane association of the other.20 To analyze the expression of Scrib, immunostaining was performed on E15.5 $Rac1^{Nkx2.5}$ hearts. Scrib is highly expressed in the myocardium surrounding the opening of the aorta in control E15.5 hearts (Fig. 4.6A). In comparison, the expression of Scrib in this area is reduced in E15.5 $Rac1^{Nkx2.5}$ heart sections (Fig. 4.6B). In addition, expression of Scrib was also reduced in
E15.5 $\text{Rac}I^{\text{Nkx2.5}}$ RV and LV myocardium compared to littermate controls (Fig. 4.6C-F). Similar to what was described in our first study,$^{19}$ Scrib protein expression is abundant in the interventricular junction in control hearts at E15.5. Overall, Scrib protein expression was significantly decreased in E15.5 $\text{Rac}I^{\text{Nkx2.5}}$ hearts compared to littermate controls (Fig. 4.6G). The loss of Scrib expression in $\text{Rac}I^{\text{Nkx2.5}}$ hearts suggests a disruption in polarity and the PCP pathway, further supporting a failure of cardiomyocytes to undergo polarization.
**Figure 4.6. Loss of Scrib expression in Rac1\(^{Nkx2.5}\) hearts.**

Scrib immunostaining was performed on E15.5 Rac1\(^{Nkx2.5}\) and Rac1\(^{ff}\) heart sections. The areas analyzed included the myocardium surrounding the aorta (A, B), RV (C, D) and the LV (E, F). The number of Scrib-expressing positive cells was significantly decreased in the myocardium surrounding the aorta (OFT), RV and LV of Rac1\(^{Nkx2.5}\) hearts (G). *P*<0.05, **P**<0.01 by Student’s t-test. Scale bars: 50 µm.

### 4.4.6 Decreased proliferation rate in Rac1\(^{Nkx2.5}\) hearts

The observed defects in Rac1\(^{Nkx2.5}\) ventricular myocardium development could also be attributed to a decrease in cell proliferation and/or aberrant apoptosis. Proliferation of the heart chambers has been shown to be highest at E9.5 in the developing mouse heart.\(^25\) Thus, proliferation of E9.5 Rac1\(^{Nkx2.5}\) hearts was analyzed by immunostaining for phospho-histone H3 (pHH3), which is a marker of the mitotic phase of cell division. The proliferation rate of the ventricular chambers was found to be significantly reduced in E9.5 Rac1\(^{Nkx2.5}\) hearts compared to littermate Rac1\(^{ff}\) controls (Fig. 4.7A-C). Along with this, cyclin D1, a marker of cell cycle progression through the G1/S phase, was significantly decreased in E9.5 Rac1\(^{Nkx2.5}\) myocardium compared to Rac1\(^{ff}\) controls (Fig. 4.7D-F). Immunostaining for cleaved caspase-3 (CC3), a marker of activated apoptosis, showed little to no apoptosis in both control E9.5 Rac1\(^{ff}\) and Rac1\(^{Nkx2.5}\) ventricular myocardium (Fig. 4.7G, H), while apoptotic cells were found in tissue outside of the myocardium (Fig. 4.7I, J). However, this data does not preclude apoptosis from potentially playing a role in later developmental time points in Rac1\(^{Nkx2.5}\) hearts. Thus, the decreased proliferation rate and expression of cyclin D1 in Rac1\(^{Nkx2.5}\)
hearts suggests a critical role for Rac1 in regulation of cardiomyocyte proliferation and cell cycle progression in the ventricular myocardium, after initial specification of cardiac progenitors.
Figure 4.7. Decreased proliferation rate in $Rac1^{Nkx2.5}$ hearts.

Phospho-histone H3 (pHH3) immunostaining to mark proliferating cells undergoing mitosis in ventricular myocardium of E9.5 $Rac1^{Nkx2.5}$ and $Rac1^{ff}$ hearts (A, B). Proliferation rate was significantly decreased in E9.5 $Rac1^{Nkx2.5}$ ventricular myocardium compared to littermate controls (C). *$P<0.05$ by Student’s t-test. Cyclin D1 immunostaining to mark cell cycle progression in ventricular myocardium of E9.5 $Rac1^{Nkx2.5}$ and $Rac1^{ff}$ hearts (D, E). Cyclin D1 was significantly decreased in E9.5 $Rac1^{Nkx2.5}$ ventricular myocardium compared to littermate controls (F). **$P<0.01$ by Student’s t-test. Cleaved caspase-3 (CC3) immunostaining to mark apoptotic cells in ventricular myocardium of E9.5 $Rac1^{Nkx2.5}$ and $Rac1^{ff}$ hearts. No apoptosis was detected in E9.5 $Rac1^{Nkx2.5}$ and $Rac1^{ff}$ ventricular myocardium (G, H). Apoptotic cells were detected elsewhere in the embryo in cells outside of the ventricular myocardium (I, J). Scale bars: 10 µm (A, B, H, I), 50 µm (D-G).

4.5 Discussion

Recent studies have implicated PCP signaling and cell polarity as a critical regulator of compact myocardium development. $Vangl2$, $Scrib$ and $Dishevelled$ mouse mutants have cardiomyocytes that are not polarized and the ventricular myocardium is thinned, resembling LVNC.26-29 $Rac1$ is a known downstream effector of PCP signaling; however the role of $Rac1$ in ventricular myocardium development is unclear.30 In the present study, we demonstrated that downregulation of $Rac1$ signaling in the ventricular myocardium disrupted formation of a trabecular network and development of the compact myocardium. In addition, $Rac1^{Nkx2.5}$ mice had a bifid cardiac apex, defects in
ventricular septum formation and OFT alignment. The organization and polarization of cardiomyocytes in the Rac1 deficient ventricular myocardium was also abnormal. Overall, proliferation was decreased in $\text{Rac1}^{\text{Nkx2.5}}$ hearts, along with expression of the PCP protein, Scrib. Our study demonstrates a critical role for Rac1 signaling during ventricular trabeculation and compact myocardium development (Fig. 4.8).

Proliferation of the heart is a regulated spatially and temporally during development. Proliferation rate of the ventricular chambers peak at E9.5 and will gradually decrease during development.\textsuperscript{25} Studies have shown that cardiomyocyte proliferation during development is the major determinant of overall cardiac size. The mass of the heart must increase to match the increasing circulatory demands of the growing embryo.\textsuperscript{31} Rac1 has been shown to regulate proliferation through various pathways. Our data showed that a Rac1 deficiency in the ventricular myocardium led to a reduced proliferation rate. However, the mechanism through which Rac1 regulates cardiomyocyte proliferation is unclear. Rac1 has been shown to regulate cyclin D1, a cell cycle regulator, through NF-κB.\textsuperscript{32, 33} Whether Rac1 signaling acts through the NF-κB pathway in the developing ventricular myocardium to regulate cell cycle progression and proliferation should be determined in future studies (Fig. 4.8).
Figure 4.8. Rac1 regulation of ventricular myocardium development.

Rac1 signaling regulates actin polymerization, leading to cardiomyocyte polarization. In addition, Rac1 regulates cardiomyocyte proliferation through cyclin D1. Overall, Rac1 signaling regulates proliferation and polarity of cardiomyocytes, leading to development of the compact myocardium and trabeculation formation.

An earlier report by Boczonadi et al also used a Nkx2.5-Cre;Rac1\textsuperscript{f/f} mouse line in their studies.\textsuperscript{20} However, the Nkx2.5-Cre mouse used by Boczonadi et al was an Nkx2.5 heterozygous mouse since the Cre recombinase gene was knocked into the Nkx2.5 genetic locus.\textsuperscript{34} In contrast, the Nkx2.5-Cre mouse used in this study is a transgenic mouse and retains homozygous expression of Nkx2.5.\textsuperscript{21} The Nkx2.5-Cre;Rac1\textsuperscript{f/f} mice used in Boczonadi et al were embryonic lethal by E13.5, which precluded analysis of ventricular septation and OFT alignment with the developing ventricles.\textsuperscript{20} The earlier lethality of the Nkx2.5-Cre;Rac1\textsuperscript{f/f} mouse line compared to the Rac1\textsuperscript{Nkx2.5} used in this
study is likely due to the heterozygous expression of \textit{Nkx2.5} compounded with decreased Rac1 signaling in the ventricular myocardium. Furthermore, whether the CHDs reported in the study by Boczonadi et al are exclusively due to deficient Rac1 signaling or also due to downregulation of \textit{Nkx2.5} is unclear. Our data only implicates Rac1 signaling and we were able to demonstrate that \textit{Rac1}^{Nkx2.5} hearts also exhibit VSDs and OFT alignment defects.

Our previous work also reported a bifid cardiac apex when \textit{Rac1} was specifically deleted in the anterior SHF and all anterior SHF-derived cells.\textsuperscript{19} We had postulated that loss of Rac1 signaling in the RV led to an inability of the nonpolarized and disorganized cardiomyocytes to bridge the interventricular junction to unify the two developing ventricles and form a distinct cardiac apex. However, from this work using the \textit{Rac1}^{Nkx2.5} hearts, we show that development of the cardiac apex is also dependent on polarization and organization of the cardiomyocytes in the LV myocardium. Interestingly, other reports where PCP signaling was disrupted did not report observations of a bifid cardiac apex, despite similar reports of cardiomyocyte disorganization.\textsuperscript{27, 28} Rac1 is a known downstream effector of PCP signaling, regulating actin cytoskeleton dynamics and cell polarity.\textsuperscript{35, 36} Our findings suggests that development of a bifid cardiac apex involves a pathway specific to Rac1 signaling. In addition, since Rac1 is a pleiotropic effector of numerous cellular events, the concomitant disruption of several cellular mechanisms are likely responsible for bifid cardiac apex, along with the other observed CHDs in \textit{Rac1}^{Nkx2.5} hearts.

Our lineage tracing analysis with the \textit{mT/mG} global double florescence mouse showed similar GFP\textsuperscript{+} expression in domains of the heart that were reported by McFadden
et al, who used a lacZ reporter. However, we also showed Cre recombinase activity in the OFT and atria, which were reported to be minimal by McFadden et al.\textsuperscript{21} This discrepancy in Cre recombinase activity may be due to the additional β-galactosidase enzymatic reaction step that is required to visualize the blue colour of lacZ staining, making it a less efficient reporter compared to a GFP reporter. These results suggest that future lineage tracing analysis should use a fluorescence reporter as a more reliable readout of expression compared to a lacZ reporter.

Rac1 is involved in reactive oxygen species (ROS) generation through activation of NADPH oxidase.\textsuperscript{37, 38} ROS mediates numerous cellular functions including proliferation, cell survival, differentiation and migration.\textsuperscript{39} The levels of ROS are finely tuned in a cell to regulate these diverse functions. For example, excess ROS induces apoptosis while basal levels of ROS regulate gene expression and proliferation.\textsuperscript{40-42} Work in our lab has shown that ROS levels must be tightly regulated to facilitate normal cardiac development. Excess ROS induced by pregestational maternal diabetes and decreased levels of ROS in the NADPH oxidase Nox2 knockout mouse have a detrimental effect on heart development, leading to a spectrum of CHDs.\textsuperscript{43, 44} Whether the CHDs observed in the $Rac1^{Nkx2.5}$ hearts can also be attributed to decreased ROS levels should be determined in future studies.

In summary, our study suggests a critical role for Rac1 regulation of cardiomyocyte organization and polarization in development of the ventricular myocardium. A $Rac1$ deficiency in the myocardium disrupts cardiomyocyte organization and proliferation, leading to bifid cardiac apex, VSDs and OFT alignment and ventricular
myocardium defects. Whether perturbed Rac1 signaling in the ventricular myocardium underlies human cases of LVNC should be further studied.

4.6 Footnotes

This work was funded by operating grants to Q.F. from Canadian Institutes of Health Research (CIHR) and Heart & Stroke Foundation of Ontario (HSFO). C.L. was supported by a Natural Sciences and Engineering Research Council (NSERC) Scholarship. Q.F. is a HSFO Career Investigator. The authors would like to thank Murong Liu for her assistance in mouse breeding and genotyping.
4.7 References


Chapter 5 Discussion

5.1 Summary of Major Findings

The overall objective of this thesis was to investigate the role of Rac1 during embryonic heart development in mice. Specifically, I studied the role of Rac1 in cardiomyocyte polarity and development of OFT, cardiac septum and the ventricular myocardium. To carry out these experiments, the Cre/LoxP system was used throughout the studies to generate a mouse model of Rac1 deficiency. More specifically, the Rac1\textsuperscript{ff} transgenic mouse was used, combined with the anterior SHF-specific Mef2c-Cre or the ventricular myocardium specific Nkx2.5-Cre transgenic mouse. The major aim of this thesis was to unravel the mechanisms of Rac1 regulation of cell polarity during embryonic heart development. Experimental approaches included \textit{in vivo} lineage tracing, histological analysis of heart sections, immunostaining, \textit{in vitro} cell cultures, \textit{ex vivo} heart explants, and biochemical and molecular assays.

In Chapter 2, I investigated the role of Rac1 in cardiomyocyte polarity and development of cardiac septum and right ventricular myocardium. To carry out this aim, the anterior SHF-specific Mef2c-Cre mouse was bred to the Rac1\textsuperscript{ff} mouse to generate a SHF-specific deletion of Rac1, Rac1\textsuperscript{SHF}. One of the distinct CHDs first noticed was a bifid cardiac apex in Rac1\textsuperscript{SHF} hearts, where the RV bifurcates from the LV instead of forming a distinct left-pointing apex. Cases of bifid cardiac apex are extremely rare in humans and are often associated with other CHDs.\textsuperscript{1,2} Whether cases of bifid cardiac apex in humans are also associated with aberrant Rac1 signaling during embryonic development remains unknown. The Rac1\textsuperscript{SHF} hearts also had other CHDs including VSDs, ASDs and defective myocardium and trabeculation formation in the RV. Further
analysis of heart cryosections showed that cardiomyocyte organization, polarity and alignment was severely disrupted in P0 \( \text{Rac1}^{\text{SHF}} \) hearts. The \( \text{Rac1}^{\text{SHF}} \) cardiomyocytes were rounded and spherical shaped compared to controls, which were elongated and aligned with each other. The actin cytoskeleton organization, expression of \( \beta \)-catenin cell-cell junctions and expression of the PCP protein, Scrib, were also disrupted in \( \text{Rac1}^{\text{SHF}} \) hearts. In addition, explant cultures of E12.5 \( \text{Rac1}^{\text{SHF};mT/mG} \) RV showed defects in migratory ability of GFP\(^+\) SHF-derived cells. \textit{In vitro} primary cardiomyocyte cultures from \( \text{Rac1}^{\text{SHF}} \) hearts displayed defects in lamellipodia formation and cardiomyocyte polarization along with a decreased cardiomyocyte axis length. Furthermore, \textit{in vivo} lineage tracing showed a deficient SHF progenitor contribution to \( \text{Rac1}^{\text{SHF}} \) hearts, likely due to defects in migratory ability of SHF progenitors. Molecular analysis demonstrated decreased expression levels of key cardiac transcription factors in E12.5 \( \text{Rac1}^{\text{SHF}} \) RV compared to littermate controls including \( \text{Nkx2.5}, \text{Gata4}, \text{Tbx5} \) and \( \text{Hand2} \). In addition, apoptosis was significantly upregulated in the RV and interventricular septum of E11.5 \( \text{Rac1}^{\text{SHF}} \) hearts with no significant difference in proliferation rate compared to littermate controls. Overall, this study showed that Rac1 is critical to embryonic heart development by regulating the actin cytoskeleton to direct cardiomyocyte polarity and migration of SHF progenitors into the developing heart.

Building upon the findings that Rac1 is crucial for cardiomyocyte polarity from the first study in Chapter 2, the experiments in \textbf{Chapter 3} analyzed the role of Rac1 in OFT development, which is known to be a process regulated by cell polarity.\(^{3, 4} \) This study demonstrated for the first time that Rac1 is critical for overall organization of the anterior SHF progenitors and OFT development. Analysis of \( \text{Rac1}^{\text{SHF}} \) embryos at E9.5 showed less SHF cells in the splanchnic mesoderm and the proliferation rate of SHF cells
was significantly reduced compared to controls. The $Rac1^{SHF}$ SHF progenitors in the dorsal pericardial wall had defects in overall cell shape and cell organization compared to littermate controls. As a result of these early defects at E9.5, the $Rac1^{SHF}$ OFT was shortened and organization of the myocardial cells was disrupted by E10.5. Furthermore, $Rac1^{SHF}$ hearts displayed a spectrum of OFT defects including double outlet right ventricle (DORV), overriding aorta, persistent truncus arteriosus (PTA), transposition of the great arteries (TGA), aortic atresia, pulmonary stenosis, vascular rings and aortic valve defects. At E12.5, $Rac1^{SHF}$ myocardial cells lacked a polarized morphology and displayed abnormalities in myocardialization and migration of cardiomyocytes into the flanking OFT cushion. Subsequently, the P0 $Rac1^{SHF}$ proximal OFT remained mesenchymal instead of muscularized. Interactions between SHF progenitors and cardiac neural crest cells have been shown to be critical for remodeling and development of the aortic arches. Sema3c expression, which acts as a chemoattractant for neural crest cells, was reduced in E10.5 $Rac1^{SHF}$ OFT myocardium. Consequently, the number of migrating cardiac neural crest cells into $Rac1^{SHF}$ hearts was reduced. Moreover, in vivo lineage tracing with the mT/mG reporter mouse showed a decreased contribution of SHF cells to the $Rac1^{SHF}$ OFT myocardium and aortic valves at E12.5 and E14.5. Lastly, intrauterine echocardiography performed at E18.5 demonstrated evidence of aortic valve regurgitation along with reduced ejection fraction and fractional shortening in $Rac1^{SHF}$ embryos. Taken together, this data suggests that Rac1 regulates SHF progenitor cell shape and organization in the splanchnic mesoderm, which is critical for overall cardiac OFT development.

In Chapter 4, the Nkx2.5-Cre transgenic mouse was used to study the role of Rac1 in development of the ventricular myocardium. Thus far, in Chapter 2 and Chapter
3 of this thesis, the Mef2-Cre mouse was used, which directs Cre recombinase activity starting at E7.5 to the anterior SHF and all anterior SHF-derived structures, including the RV, interventricular septum and OFT. The Cre recombinase in the Nkx2.5-Cre transgenic mouse is activated after initial specification of cardiac progenitors at E8.5 but not in earlier cardiac progenitors and is also active in the developing LV, along with the RV myocardium. Mice with a Rac1 deficiency in the ventricular myocardium, Rac1<sup>Nkx2.5</sup>, also had bifid cardiac apex, similar to what was observed in Chapter 2 with the Rac1<sup>SHF</sup> hearts. Other CHDs found in Rac1<sup>Nkx2.5</sup> hearts included VSDs, defects in trabeculation formation and a significantly thinner compact myocardium. Rac1<sup>Nkx2.5</sup> hearts also had misalignments of the OFT with the ventricles, leading to DORV and overriding aorta. Actin filament formation was severely disrupted and cardiomyocytes failed to undergo polarization in Rac1<sup>Nkx2.5</sup> hearts. Proliferation of the myocardium in E9.5 Rac1<sup>Nkx2.5</sup> hearts was significantly reduced compared to littermate controls, while no significant apoptosis was observed at this time point. In addition, expression of the PCP protein, Scrib, was reduced in E15.5 Rac1<sup>Nkx2.5</sup> hearts compared to littermate controls.

Overall, our study suggests a critical role for Rac1 regulation of myocardial organization and polarization. Whether perturbed Rac1 signaling in the ventricular myocardium is related to cases of left ventricular noncompaction (LVNC) defects reported in human patients remains to be determined.

In summary, I have demonstrated a crucial role for Rac1 in embryonic heart development. More specifically, I showed that Rac1 is critical for development of cardiomyocyte polarity and a deficiency in Rac1 leads to defects in cardiomyocyte polarization. Next, I showed that Rac1 is crucial for organization of the anterior SHF progenitors in the dorsal pericardial wall and overall OFT development. Lastly, I showed
that Rac1 is essential for ventricular myocardium development and a Rac1 deficiency leads to trabecular defects and noncompaction of the myocardium. My studies provide novel insights into the developmental mechanisms that may be disturbed in some cases of human CHDs and may provide information for improved diagnostics and therapies. A summary of the findings from these studies is shown in Figure 5.1.

**Figure 5.1. Summary of the cellular mechanisms by which Rac1 regulates embryonic heart development.**

Rac1 signaling regulates various mechanisms of heart development including proliferation, actin polymerization, gene expression and cell survival, leading to cardiomyocyte polarity, development of the OFT, cardiac septum and the ventricular myocardium.
5.2 Study Limitations

5.2.1 Mouse models of CHDs

Mouse models have been used extensively to understand physiology and pathophysiology, including the mechanisms of normal heart development and how CHDs arise in humans. The cardiac developmental events and anatomy of the heart are very similar in mouse and humans. Since the mouse genome was first sequenced in 2002, it has been shown that approximately 99% of mouse genes are homologous to human genes. This makes the mouse an excellent mammalian model for comparative genomics and a powerful tool to help us further understand cardiogenesis. In addition, manipulations to the mouse genome are relatively straightforward and specific, making it an invaluable model to study the mechanisms of CHDs. However, despite great advancements to research that the genetically modified mouse has provided, use of the mouse model in some cases does not always completely recapitulate the CHDs observed in humans due to distinct physiological differences and mouse genetic backgrounds. For example, in mutant mice with one copy of Tbx5, the CHDs that arise accurately reflect those found in Holt-Oram Syndrome (HOS) patients with a heterozygous mutation of Tbx5. On the other hand, deletion of one copy of Nkx2.5 in mice only recapitulates some aspects of the CHDs found in humans with an Nkx2.5 deficiency. This indicates that specific genes may have differing dosage sensitivities between mouse and humans. In addition, polymorphisms or mutations of a gene in humans often present as a reduction in the protein level and/or activity. The associated phenotypes with these human mutations may not always be accurately reflected in a genetic knockout mouse. Furthermore, different genetic modifiers such as epigenetics can influence phenotypical expression of genetic mutations in different mouse strains. For example, mutations in
*Gata4* in the C57BL6/J strain have an increased frequency of CHDs compared to the FVB/NCrI strain. In addition to potential genetic variabilities in mouse models, differences in physiology such as a shorter gestation time in mouse and a faster heart rate compared to humans could also influence the phenotypes that arise in mouse mutants. Overall, the use of mouse models will continue to be a vital tool in medical research but their inherent limitations must be considered when they are employed to study CHDs.

Conventional gene targeting, where a specific gene is completely ablated in all cells of a mouse, has been a critical genetic tool to elucidate gene function. However, approximately 30% of these knockout mice are embryonic lethal or have early postnatal lethality, which precludes analysis at later time points. To avoid this issue, the *Cre/loxP* system has been used extensively to allow spatial control of genetic deletions. *Lox/P* sequences flank a critical gene region and act as recognition sites for Cre recombinase, which is under regulatory control of a tissue specific promoter. The *Rac1* knockout mouse is embryonic lethal, therefore the *Cre/loxP* system was used extensively throughout this thesis to specifically delete *Rac1* in the anterior SHF and the ventricular myocardium. The heart is comprised of different progenitors that give rise to numerous cell types including cardiomyocytes, fibroblasts, epicardial cells and endothelial cells. The use of the *Cre/loxP* system allows tracking, isolation and characterization of specific cardiac progenitors and their derivatives, thus facilitating our understanding of heart development. However, the *Cre/loxP* system does present with potential limitations that must be considered. Some studies have reported Cre recombinase toxicity in transgenic mice. There have also been reports of phenotypical abnormalities and altered gene expression at the sites surrounding *Cre* insertion. To reduce the potential of Cre toxicity, the transgene should stay as a single copy as it is passed onto future generations.
Another issue with the Cre/loxP system is expression of Cre in other unintended tissues. With Cre driven lineage tracing, a reporter gene (ex. GFP, lacZ) is expressed once the gene controlling Cre expression is turned on. All daughter cells derived from the first cell expressing the Cre will also be labeled. However, this may not be a true lineage tracing of a progenitor cell since any cell type with that particular Cre-controlling gene expressed will also be labeled. For example, the Isl1-Cre mouse has been used to study the SHF. However, the Isl1-Cre transgene is also expressed in tissues outside of the cardiac progenitor population, including motor neuron cells and the dorsal root ganglia.\textsuperscript{20} Recently, a study showed that Isl1-Cre also marks a subset of cardiac neural crest cells, revealing a need for more specific cardiac progenitor markers or the use of multiple or alternative tissue-specific Cre drivers within a study. The anterior SHF Mef2c-Cre transgene was found to not overlap with cardiac neural crest cells.\textsuperscript{21}

Another issue with some Cre/loxP mouse models is whether the Cre is transmitted from the male or female parent. In this study with the Mef2c-Cre transgenic mouse, the Cre was always inherited from the male parent. The Mef2c-Cre is also active in the female germline and female inheritance would have led to full body expression of Cre recombinase in the offspring. Moreover, another potential limitation to the Cre/loxP system is that some floxed sequences may be more easily recombined than others based on the distance between loxP sites and chromatin state of the loxP sites.\textsuperscript{22} In this study, the same line of Rac1\textsuperscript{f/f} and mT/mG transgenic mice were used for all experiments, therefore the efficiency of recombination should be similar for all samples. Another limitation to the Cre/loxP system is the inability to control the level or efficiency of Cre activity. For example, the Mef2c-Cre transgenic mouse was able to sufficiently downregulate Rac1 expression by 51% while the Nkx2.5-Cre mouse was only able to do
so by 35%. This inability to control the level of Cre activity can be a limitation when trying to recapitulate CHDs based on human genetic mutations that present with dosage sensitivities. Nevertheless, despite the inability to regulate Cre efficiency, the phenotypes shown using the \textit{Cre/loxP} system with the \textit{Mef2c-Cre} and \textit{Nkx2.5-Cre} transgenic mouse in this thesis allowed us to elucidate the role of Rac1 in embryonic heart development, furthering our basic understanding of the mechanisms that could underly CHDs that arise in humans. Whether human patients have deficiencies in Rac1 expression and/or activity that lead to development of CHDs remains to be determined.

5.2.2 Use of \textit{in vitro} cell culture systems

In Chapter 2 of this thesis, \textit{in vitro} cell cultures and explants were used to understand the role of Rac1 in cardiomyocyte polarity. Specifically, E12.5 RV from \textit{Rac1^{SHF};mT/mG} and littermate controls were explant cultured onto collagen to study migration of GFP$^+$ SHF-derived cells. This allowed us to visualize and monitor the migratory ability of SHF-derived cells in a live manner. In addition, primary cardiomyocytes from WT hearts were isolated and treated with Ad-Rac1N17 to study lamellipodia formation after a scratch injury. Primary cardiomyocytes from P0 \textit{Rac1^{SHF}} RV were also cultured to analyze cellular characteristics such as length and shape. All of these \textit{in vitro} techniques allowed us to examine the role of Rac1 in cardiomyocyte polarity and embryonic heart development, which would have been difficult to do in the \textit{in vivo} model. A limitation to the use of \textit{in vitro} cell cultures is that it does not fully simulate the \textit{in vivo} environment that the cells would normally reside in. Cells in an \textit{in vivo} model are under constant nourishment from the surrounding blood vessels, have interactions with other cardiac cell types in a 3D environment and undergo biomechanical forces due to the beating movements of the heart, which cannot be fully replicated in the \textit{in vitro} setting.
Furthermore, use of the adenovirus, Ad-Rac1N17, to knock down Rac1 expression in WT primary cardiomyocyte cultures likely does not replicate the deficiency in Rac1 expression found in Rac1SHF hearts. However, culture of WT cardiomyocytes was necessary since primary culture of cardiomyocytes from Rac1SHF RV did not form a confluent cell layer to facilitate a scratch assay due to the inability of many of the Rac1SHF cardiomyocytes to adhere to the culture plate. Despite the limitations that in vitro cultures present, use of these techniques in this thesis were crucial to help further delineate the specific role of Rac1 in cardiac progenitor migration and cardiomyocyte polarity during cardiac development. Overall, these in vitro experiments act as supporting data for the in vivo findings in Chapter 2.

5.3 Suggestions for Future Research

This thesis demonstrated a critical role for Rac1 in embryonic heart development, specifically in cardiomyocyte polarity, SHF progenitor cellular organization and development of the OFT and ventricular myocardium. However, some aspects of Rac1 signaling should be investigated in future studies to gain a further understanding of the role of Rac1 in cardiogenesis.

In Chapter 2 and Chapter 4 of this thesis, I showed that a deficiency in Rac1 leads to defects in ventricular myocardium development. The trabeculation of Rac1SHF RV and Rac1Nkx2.5 RV and LV are poorly formed and the compact myocardium is thinner compared to littermate controls. It would be of interest to study cardiac conduction system development in these hearts as well. Parts of the conduction system are derived from the embryonic myocardium that retain a primitive phenotype while the surrounding myocardial cells developing into ventricular myocardium. Therefore, I would predict
that defects in ventricular myocardium development would likely lead to defects in cardiac conduction system development as well. Cardiac neural crest cells have been shown to have a role in the development and maturation of the conduction system. Ablation of cardiac neural crest cells in chick embryos result in noncompaction of the conduction system bundles. In mouse lineage tracing studies, cardiac neural crest cells have been shown to contribute to formation of the cardiac conduction system. In addition, SHF progenitors have also been demonstrated to contribute to development of the cardiac conduction system. The sinal atrial node and conduction system of the RV are derived from the SHF, along with a small subset of cells in the atrial-ventricular node, His Bundle and left bundle branches. Whether Rac1 plays a role in development of the cardiac conduction system remains to be determined. Future studies using the Rac1\textsuperscript{SHF} mouse to study the conduction system would be particularly interesting since I would predict the conduction system to be malformed in the RV. Furthermore, since cardiac neural crest cells also contribute to the conduction system, the LV conduction system may also be defective in Rac1\textsuperscript{SHF} hearts. However, whether SHF cells signal to cardiac neural crest cells through semaphorin-plexin signaling during conduction system development is currently unknown.

In addition to the cardiac conduction system, development of the coronary arteries could also be analyzed in future studies. The defects observed in myocardium development in Chapter 2 and Chapter 4 likely lead to defects in coronary artery formation as well. Coronary artery development depends on complex crosstalk signaling between the epicardium and the myocardium, mediated in part by growth factors including fibroblast growth factor (FGF), platelet derived growth factor (PDGF) and angiopoietin signaling. Whether this crosstalk signaling and coronary artery formation
is also disrupted in $Rac^{'Nkx2.5}$ and $Rac^{'SHF}$ hearts remains unknown. Interestingly, disruption of the PCP pathway in homozygous Looptail ($Lp$) mutant mice displayed defects in coronary artery development, despite the lack of expression of Vangl2 in the epicardium and epicardial-derived cells. Thus, the coronary artery defects were attributed to abnormal development of the adjacent myocardial cells.$^{28}$

In **Chapter 3**, I showed that loss of Rac1 in SHF progenitors in $Rac^{'SHF}$ hearts also lead to defects in aortic valve maturation and remodeling. P0 $Rac^{'SHF}$ aortic valves are thickened with a reduced cell density compared to littermate controls, which have thin elongated aortic valve leaflets. Aortic valve development involves ECM remodeling and Rac1 has been shown to regulate the ECM remodeling process in the eye and the knee.$^{29-31}$ Whether Rac1 plays a similar role during the process of aortic valve development and remodeling in cardiogenesis is unknown. Future studies could use specific Cre recombinase transgenic mice that target specific cells in the valves to study the role of Rac1, such as the endothelial $Tie2$-Cre mouse.$^{32}$

In **Chapter 2**, I showed that expression of some key cardiac transcription factors were downregulated in the E12.5 $Rac^{'SHF}$ RV. Rac1 has been shown to promote gene expression through NF-$\kappa B$ and/or JNK signaling.$^{33,34}$ Another study has shown that Rac1 promotes nuclear accumulation of $\beta$-catenin in the canonical Wnt pathway.$^{35}$ Whether Rac1 acts through these above mentioned pathways or others to regulate gene expression should be determined in future studies. Furthermore, it would be of interest to determine whether the same cardiac transcription factors that are downregulated in $Rac^{'SHF}$ hearts are also downregulated in $Rac^{'Nkx2.5}$ hearts from **Chapter 4**. The developing RV and LV have distinct gene regulatory programs. For example, the transcription factor Hand1 is
mostly expressed in the LV and loss of Hand1 results in LV defects and dysregulation of downstream gene expression. The transcription factor Hand2 is mostly expressed in the SHF and loss of Hand2 results in RV defects, along with defects in the OFT.\textsuperscript{36} Although Hand1 and Hand2 have partially overlapping roles in ventricular development, their individual expression patterns in the developing heart suggests existence of a distinct gene regulatory program between the RV and LV.\textsuperscript{8} Therefore, whether loss of Rac1 in the LV of Rac1\textsuperscript{Nkx2.5} hearts lead to dysregulation of the same genes in the Rac1\textsuperscript{Nkx2.5} RV should be further studied.

Rac1, along with other Rho GTPases, have a tight spatio-temporal level of regulation. For example, loss of Rac1 in embryonic hematopoietic stem cells does not affect proliferation rate, in contrast to adult hematopoietic stem cells, where the proliferation rate is decreased.\textsuperscript{37,38} In Chapter 2 of this thesis, which used the Rac1\textsuperscript{SHF} mouse, Rac1 was found to regulate cell survival in the ventricular myocardium at E11.5 and no differences were observed in proliferation rate of the myocardium. However, at an earlier developmental time point in Chapter 4, which used the Rac1\textsuperscript{Nkx2.5} mouse, Rac1 was found to regulate proliferation of the E9.5 ventricular myocardium with no effect on cell apoptosis. Proliferation of the mouse heart is highest at E9.5 and will gradually decrease as development proceeds.\textsuperscript{39} Our results suggest a temporal regulation of Rac1 control of proliferation in the ventricular myocardium, where Rac1 plays a critical role during the highest growth rate at E9.5. Although proliferation of the ventricular myocardium continues, Rac1 does not seem to play a critical role by E11.5. Similarly, Rac1 control of cell survival also seems to be regulated in a temporal manner in the ventricular myocardium. However, whether the temporal differences observed in this thesis were due to differences in the level of Rac1 downregulation remain to be
determined. Specifically, in Chapter 2, the *Mef2c-Cre* transgenic mouse was used while in Chapter 4, the *Nkx2.5-Cre* mouse was used, and differences in Cre recombinase activity likely exist between these two transgenic mouse lines. To definitively determine the temporal pattern of Rac1 activity, the same Cre recombinase transgenic mouse should be used for analysis. In addition, a tamoxifen inducible Cre transgenic mouse could be used to delete Rac1 at specific developmental time points. Lastly, further studies will be necessary to determine the upstream signals, such as growth factors, hormones, cytokines and/or adhesion molecules that temporally regulate Rac1 in development of the heart.

Finally, in Chapter 4, proliferation was significantly decreased in the *Rac1*Nkx2.5 ventricular myocardium. However, the specific mechanisms that Rac1 act through to regulate proliferation during cardiac development is unclear. Rac1 signals through NF-κB to regulate cyclin D1, the cell cycle regulator. Furthermore, the D-type cyclins have been shown to be critical regulators of cardiomyocyte proliferation during normal heart development. Future studies will be performed to determine whether this is the pathway that Rac1 acts through to regulate cardiomyocyte proliferation.

5.4 Conclusion

This thesis is the first to provide evidence of a critical role for Rac1 signaling in embryonic heart development. Here I demonstrated the importance of Rac1 signaling in cardiomyocyte polarity, development of OFT and cardiac septum, and formation of the ventricular myocardium. Deficient Rac1 signaling disrupts several developmental pathways including proliferation, cell survival, gene expression and actin dynamics, leading to a spectrum of CHDs including a bifid apex, ASDs, VSDs, OFT defects and ventricular myocardium defects. While further research is required to determine whether
Rac1 mutations are involved in human patients with CHDs, this thesis provides evidence that aberrant Rac1 signaling may underlie some cases of this disease. In addition to potential mutations in the Rac1 gene in humans, aberrant signaling of this pathway may also be due to mutations in the GEFs and GAPs that regulate Rac1. For example, mutations in the GEF, Son of sevenless homolog 1 (Sos1) is found in 10-15% of Noonan syndrome patients, who present with a range of developmental defects including CHDs. Sos1 acts as a bifunctional GEF for Ras and Rac and a missense mutation in E846K leads to constitutive activation. A study using a mutant mouse model of Noonan syndrome showed CHDs along with other birth defects, which could be ameliorated with prenatal administration of a MEK inhibitor. Whether this type of treatment can be applied to human patients should be further studied. Overall, the work in this thesis has provided valuable insight into the mechanisms by which Rac1 regulates cardiomyocyte polarity and embryonic heart development. My research has broadened our understanding of the developmental mechanisms regulated by Rac1 and may have implications in future diagnosis or treatment of CHDs.
5.5 References


36. Thomas T, Yamagishi H, Overbeek PA, Olson EN, Srivastava D. The bHLH factors, dHAND and eHAND, specify pulmonary and systemic cardiac ventricles independent of left-right sidedness. *Dev Biol.* 1998;196:228-236.


Appendix

Qingping Feng - eSirius Notification - Annual Protocol Renewal APPROVED by the AUS
2007-011-03::7

From: [Redacted]
To: [Redacted]
Date: 03/12/2014 8:59 AM
Subject: eSirius Notification - Annual Protocol Renewal APPROVED by the AUS 2007-011-03::7
CC: [Redacted]

Western

2007-011-03::7:

**AUP Number:** 2007-011-03  
**AUP Title:** Modulation of Myocardial Function in Myocardial Infarction, Sepsis, anemia and Diabetes

**Yearly Renewal Date:** 12/01/2014

The YEARLY RENEWAL to Animal Use Protocol (AUP) 2007-011-03 has been approved, and will be approved for one year following the above review date.

1. This AUP number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this AUP number.
3. Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.

**REQUIREMENTS/COMMENTS**
Please ensure that individual(s) performing procedures on live animals, as described in this protocol, are familiar with the contents of this document.

The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

Submitted by: Kinchlea, Will D  
on behalf of the Animal Use Subcommittee
Curriculum Vitae

Carmen Leung

Post-secondary Education and Degrees:
The University of Western Ontario
London, Ontario, Canada
2005-2009
BMSc, Honors Specialization in Physiology

The University of Western Ontario
London, Ontario, Canada
2009-2015
PhD, Physiology & Collaborative Program in Developmental Biology

Honours and Awards:
University of Western Ontario Scholarship of Excellence
2005

Robert & Ruth Lumsden Undergraduate Award in Science
The University of Western Ontario
London, Ontario
2006

Queen Elizabeth II Aiming for the Top Scholarship
The University of Western Ontario
London, Ontario
2005-2009

Dean's Honour List, Graduation with Distinction
The University of Western Ontario
London, Ontario
2005-2009

NSERC Alexander Graham Bell Canada Graduate Scholarship
The University of Western Ontario
London, Ontario
2010-2011

Western Graduate Thesis Research Award
2011

Western Research Graduate Scholarship
2009-2014
Physiology & Pharmacology Research Day – 1st Place Poster Award (Category: Cell & Developmental Biology)
2012

London Health Research Day – 1st Place Poster Award (Category: Cardiovascular & Respiratory Health)
2013

CIHR Canadian Student Health Research Forum – Poster Competition Gold Award
Winnipeg, Manitoba
2013

Physiology & Pharmacology Research Day – 1st Place Poster Award (Category: Cell & Developmental Biology)
2013

Physiology & Pharmacology Research Day – 2nd Place Poster Award (Category: Cell & Developmental Biology)
2014

London Health Research Day – Student Choice Award
2015

**Invited Oral Presentations:**
Cardiac Biology Journal Club
London, Ontario
2013

Developmental Biology Research Day
London, Ontario
2014

Advances in Cardiovascular Medicine Symposium
London, Ontario
2014

2nd Joint Western – Nanjing Medical University Research Symposium
London, Ontario
2015

**National & International Conference Poster Presentations:**
Great Lakes Mammalian Development Conference
Toronto, Ontario
2011 & 2012

Experimental Biology
Washington, DC
2011

Weinstein Cardiovascular Development Conference
Chicago, Illinois
2012

Weinstein Cardiovascular Development Conference
Tucson, Arizona
2013

Weinstein Cardiovascular Development Conference
Madrid, Spain
2014

**Related Work Experience:**

Teaching Assistant – Introduction to Pharmacology &
Therapeutics 2060B
The University of Western Ontario
2011-2012

Teaching Assistant – Cardiovascular Pharmacology 4320A
The University of Western Ontario
2012-2013

Part-time Supplies Associate
Thermo Fisher Scientific
London, Ontario
2013-2014

**Publications:**

**Leung C,** Lu X, Liu M and Feng Q. Rac1 signaling is critical to cardiomyocyte polarity and embryonic heart development. *J Am Heart Assoc.* **2014;3:e001271.**

**Leung C,** Liu Y, Lu X, Kim M, Drysdale TA and Feng Q. Rac1 signaling is required for anterior second heart field cellular organization and cardiac outflow tract development. *J Am Heart Assoc.* **2015.**