RGS4 Expression is Associated with Spontaneously Beating Cardiomyocytes during Sinoatrial Node Development

By

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A thesis submitted in conformity with the requirements for the degree of Masters of Science

Department of Physiology

University of Toronto

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ABSTRACT

RGS4 is highly expressed in the SAN and is an important regulator of parasympathetic signaling in those cells. Our study set out to determine the utility of RGS4 expression as a marker of SAN myocyte progenitors during cardiac development. In the intact RGS4-LacZ mouse, RGS4 expression was observed in the SAN region starting at E11.5 and its expression remains strong in the SAN throughout cardiac maturation. In mESC-derived cardiomyocytes, RGS4 expression was observed to be associated in the contractile regions of the monolayer. When a RGS4-GFP transgenic mESC line was used to select for differentiated cardiomyocytes with high RGS4 expression, this cell population showed many similarities to SAN myocytes. Specifically, they expressed the pacemaker channel, HCN4, and showed spontaneous action potentials that were characteristic of SAN myocytes. Taken together these data suggest that RGS4 reporter expression may be used to select for SAN myocyte-like cells during cardiomyocyte differentiation from mESCs.
Acknowledgements

First and foremost I would like to thank my supervisor Dr. Scott Heximer for his continued support and guidance. You have not only been a wonderful mentor, but also a friend throughout my journey as a Masters student. Your confidence and endless patience with me has allowed me to mature as a student and as an individual. I am grateful to have been a part of the Heximer Lab and it has been an experience that I will always cherish.

Many thanks go to my supervisory committee: Dr. Steffen-Sebastian Bolz and Dr. Michelle Bendeck. Your guidance, support and expertise has aided in the completion of my project and thesis.

Special thanks go to the Heximer Lab: Jenny, Guillaume, Carlo, Kaveesh and Sorana. Thank you all for making life interesting and fun while in the lab. It wouldn’t have been the same without any of you.

To the Keller Lab, with all the time I’ve spent in your lab, you have made me feel truly a part of the lab. Extra special thanks are needed to Nicole, you taught me everything I know about stem cells and my project would not have been the same without you. Not only did we have an awesome collaboration, I have gained a friend out of this.

Thanks and words of appreciation are not enough to express my gratitude to my parents who have provided me with the ultimate level of support educationally and personally. Your unwavering love and belief in me has allowed me to become the person that I am today. I am truly grateful and proud to have you as my parents.

And last but definitely not least, I thank my fiancé Louis for his endless love, comfort and support. This journey would not have been possible without you.

GO RGS!!
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<td>Ab</td>
<td>Antibody</td>
</tr>
<tr>
<td>AC</td>
<td>Adenylyl cyclase</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenosine monophosphate</td>
</tr>
<tr>
<td>APC</td>
<td>Allophycocyanin</td>
</tr>
<tr>
<td>AR</td>
<td>Adenosine receptor</td>
</tr>
<tr>
<td>ATR</td>
<td>Angiotensin II receptor</td>
</tr>
<tr>
<td>AVN</td>
<td>Atrioventricular node</td>
</tr>
<tr>
<td>βAR</td>
<td>β-adrenergic receptor</td>
</tr>
<tr>
<td>bFGF</td>
<td>Basic fibroblast growth factor</td>
</tr>
<tr>
<td>BMP4</td>
<td>Human bone morphogenic protein 4</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>cTNT</td>
<td>cardiac Troponin T</td>
</tr>
<tr>
<td>Cx</td>
<td>Connexin</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>E</td>
<td>Embryonic day</td>
</tr>
<tr>
<td>EB</td>
<td>Embryoid body</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ESCs</td>
<td>Embryonic stem cells</td>
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<tr>
<td>FACS</td>
<td>Fluorescent activated cell sorting</td>
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<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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<tr>
<td>FLK-1</td>
<td>Fetal liver kinase 1</td>
</tr>
<tr>
<td>GAIP</td>
<td>Go-interacting protein</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase activating protein</td>
</tr>
<tr>
<td>GDP</td>
<td>guanosine diphosphate</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
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<tr>
<td>G-proteins</td>
<td>Guanine nucleotide-binding regulatory proteins</td>
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<tr>
<td>GPCR</td>
<td>G protein coupled receptors</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanine 5’ triphosphate</td>
</tr>
<tr>
<td>hDkk</td>
<td>human Dickkopf-1</td>
</tr>
<tr>
<td>HCN</td>
<td>Hyperpolarization-activated cyclic nucleotide-gated</td>
</tr>
<tr>
<td>IMDM</td>
<td>Iscove’s modified Dulbecco’s medium</td>
</tr>
<tr>
<td>IRES</td>
<td>Internal ribosomal entry site</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilo-daltons</td>
</tr>
<tr>
<td>LIF</td>
<td>Leukemia inhibitory factor</td>
</tr>
<tr>
<td>M2R</td>
<td>M2 muscarinic receptor</td>
</tr>
<tr>
<td>mAB</td>
<td>Mouse antibody</td>
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<td>mESCs</td>
<td>Mouse embryonic stem cells</td>
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<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffer saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chase reaction</td>
</tr>
<tr>
<td>PdgfR</td>
<td>Platelet derived growth factor receptor</td>
</tr>
<tr>
<td>PE</td>
<td>Phycoerythrin</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PKA</td>
<td>cAMP-dependent protein kinase A</td>
</tr>
<tr>
<td>PKG</td>
<td>cGMP dependent protein kinase</td>
</tr>
<tr>
<td>qPCR</td>
<td>Real time quantitative polymerase chase reaction</td>
</tr>
<tr>
<td>RBD</td>
<td>Ras-binding domain</td>
</tr>
<tr>
<td>RGS</td>
<td>Regulator of G-protein signaling</td>
</tr>
<tr>
<td>RI</td>
<td>p160-Rho associated coiled kinase inhibitor</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>SAN</td>
<td>Sinoatrial node</td>
</tr>
<tr>
<td>SAP</td>
<td>Shrimp alkaline phosphatase</td>
</tr>
<tr>
<td>SB</td>
<td>SB-431542; Inhibitor of activin receptor-like kinase receptors</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SF</td>
<td>Serum-free</td>
</tr>
<tr>
<td>TBP</td>
<td>Troponin binding protein</td>
</tr>
<tr>
<td>TTX</td>
<td>Tetrodotoxin</td>
</tr>
<tr>
<td>UTV</td>
<td>Universal transfer vector</td>
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<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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Contributions

**Stem cell studies**

These studies were performed in collaboration with the Gordon Keller lab. Lab resources and protocols were kindly provided by the Keller lab. Significant contributions from Dr. Nicole Dubois included training me with stem cell differentiation, aid in forming the project as well as troubleshooting the project.

**Electrophysiology studies**

These studies were performed in collaboration with Dr. Peter Backx’s lab. Dr. Brian Panama performed the patch clamping experiments to measure the action potentials from cells sorted for RGS4-GFP.
Chapter 1

Introduction

Cardiovascular disease is currently the number one cause of death globally. By 2030, it is estimated that 23.6 million individuals will die from cardiovascular disease\(^1\). It has been projected that cardiovascular disease will continue to remain as the single leading cause of death. Heart failure and cardiac arrhythmias are common disorders affecting the frequency of the cardiac cycle. G-protein coupled receptors (GPCRs) are ubiquitously expressed in the cardiovascular system and play an important role in the physiological and developmental regulation of the cardiovascular system\(^2\). The regulators of G-protein signaling (RGS) proteins that tightly regulate the activity of GPCRs by attenuating their signal are thought to be important in directing proper physiologic responses in the cardiovascular system\(^2\). Accordingly, increasing our understanding of the molecular and cellular mechanisms related to GPCR and RGS signaling in the cardiovascular system may uncover novel therapeutic strategies for the treatment and prevention of cardiovascular disease.

1.a. Cardiovascular development

1.a.i. Overview

The first organ to form during the development of vertebrates is the heart. It has a vital role in development by distributing nutrients and oxygen to the developing embryo\(^4\). A series of developmental steps occur before the formation of the cardiovascular system. Immediately following implantation, the mouse embryonic tissue increases in volume from extensive cell proliferation to form the extraembryonic ectoderm, the inner cell mass, and the
trophectoderm. Subsequently, the inner cell mass epithelializes into a layer of epiblast cells and the formation of the central cavity called the proamniotic cavity. The embryo at this stage is called the egg cylinder and is made up of two cell layers, the inner epiblast and the outer visceral endoderm. The outer surface of the visceral endoderm will give rise to the ventral side of the embryo and the surface of the epiblast will become the dorsal side of the embryo.

Gastrulation is the key step in configuring the body plan in vertebrates and is initiated by the formation of the primitive streak in the region of the epiblast where it will ultimately develop into the posterior end of the embryo. Morphogenic movements coupled to cell proliferation and differentiation gives rise to gastrulation and the formation of the three germ layers: ectoderm, mesoderm and endoderm during gastrulation. At the end of gastrulation, cell populations situated in different germ layers required for the formation of organs are physically brought together. This illustrates the inductive interactions that are crucial for lineage specification and tissue patterning for organ formation. The temporal and spatial segregation of cell fates in different regions of the primitive streak in combination with cell populations immediately adjacent to the primitive streak generate different signaling environments which are crucial for lineage specification. It should be noted that the precise regulation and pathways required for primitive streak and germ layer formation are not fully understood though studies have shown that this dynamic process is partially controlled by activation and inhibition of the Wnt, Nodal and BMP signaling pathways.

Myocardial cells, derivatives of the mesoderm, arise from the primitive streak during gastrulation. Cardiac progenitor cells have been shown to emerge from the anterior region of the primitive streak. However, at the primitive streak stage these cells are not fully
committed to the cardiac fate. It is at this point that the earliest markers, transcription factors MESB1 and MESP2, for cardiac progenitors are expressed. The expressions of these two markers are required for the subsequent step where the cells migrate in an anterior-lateral direction to just under the head folds where they form a cardiac crescent \(^7,14\). Determined from cell lineage studies, descendants from the cells expressing MESP1 and MESP2 eventually colonize the whole myocardium\(^{14}\). The first detection of myocardial markers arises from cells found in the cardiac crescent where myocardial differentiation is initiated. Here the activation of the key myocardial regulatory genes, NKX2.5 and GATA4, occurs in the presence of positive signaling by bone morphogenic proteins (BMPs) and fibroblast growth factors (FGFs) and the repression of wingless-related MMTV integration sites (WNTs)\(^{15}\). Fusion at the midline of the cardiac crescent forms the early heart tube. The step crucial for the formation of the heart to ensure proper orientation of ventricles and proper alignment of the heart chamber to the vasculature is the rightward looping of the heart tube\(^{16}\). Concurrently, the posterior region of the heart tube moves anteriorly and, with expansion of the myocardium, leads to the formation of cardiac chambers\(^4\).

Although the formation of cardiac chambers does not occur until after cardiac looping, it has been demonstrated that the cell fates are genetically programmed at an earlier stage\(^{16}\). Each cardiac chamber differs morphologically, has different contractile properties and has different gene patterns; however, it is unknown how chamber identities are established during development.
1.a.ii. Structures of the mouse sinoatrial node

The sinoatrial node (SAN) is embedded in the intercaval region near the entrance of the right atrium. The structure of the SAN is often described as a “comma” with the “head” located on the superior caval vein-atrial border and the “tail” located along the crista terminalis. The cells of the SAN initiate and coordinate electrical signals generating the contractions of the heart\textsuperscript{17,18}. The SAN myocytes generates an impulse which propagates through the atrial myocardium. This impulse is delayed at the atrioventricular node (AVN) and proceeds with further propagation to the ventricular myocardium via the atrioventricular bundle, bundle branches, and the Purkinje fiber network\textsuperscript{17}. The SAN is the primary pacemaker and in the case of SAN failure or atrioventricular blockage, the need for electrical conductance in the heart is backed up by the atrioventricular node which acts as the secondary/accessory pacemaker to ensure repetitive ventricular contractions. Protection to the SAN is also present—the SAN is functionally protected from the resistive load of the atrium by a surrounding area of tissue that contains myocardial phenotype but contains the expression profile of both the SAN and the working atria. This protective set of cells is further encompassed by an outer ring of connective tissue and arteries for additional protection of the SAN\textsuperscript{19-21}.

Due to the distinct electrophysiological properties of the SAN, various SAN-specific markers have been identified. The activity of the SAN requires a high level of intercellular resistance to protect it from the hyperpolarizing effects of the atrium. Given that gap junctions regulate intercellular conduction velocities, the SAN expresses a set of gap junction channels with extremely low conductance: Cx45, CX30.2, and Cx30 subunits\textsuperscript{22-24}. In contrast, the surrounding atrial working myocardium is characteristically fast-conducting and
therefore expresses the high-conductance gap junction channels Cx40 and Cx43\textsuperscript{19;20;23;25}.

Another marker that distinguishes the SAN from the atria is Scn5a, a sodium channel which is highly expressed in the atrial working myocardium and has low expression in the SAN. It has high expression in the atrial working myocardium results in rapid depolarization and conduction seen in the atria\textsuperscript{26;27}. Consequently a profile with high expression of Cx45, Cx30.2, and Cx30 along with low expression of Cx40, Cx43 and Scn5a provides a useful marker set representing both adult and fetal SAN tissue.

A key marker of the SAN is the hyperpolarization-activated cyclic nucleotide-gated cation channel 4 (HCN4), which is enriched in pacemaker tissues\textsuperscript{19;28-30}. HCN4 is responsible for the regulation of the hyperpolarization current, I\textsubscript{f}, which is required for the pacemaking potential of the SAN. The elucidation of the role of HCN4 in the SAN will be discussed below in the discussion of the electrophysiological properties of the SAN. HCN4 is regulated by NKX2.5 expression, where it represses the expression of HCN4. This was demonstrated in NKX2.5 deficient embryos which showed a significant ectopic expression of HCN4 in the heart\textsuperscript{31}. HCN4 expression is initiated by unknown factors as the heart matures, but upon the expression of NKX2.5 during development HCN4 expressions ceases in many areas confining its expression to NKX2.5 negative venous poles.

The T-box transcription factor TBX3 is expressed in the developing as well as mature conduction system including the SAN. TBX3 is a transcriptional repressor whose expression delineates the sinoatrial node region\textsuperscript{27}. There is a lineage segregation of differentiating cardiomyocytes where TBX3-negative cells are precursors for atrial cells and TBX3-positive expressing cells are precursors for sinoatrial node cells. Hoogaars \textit{et al.}\textsuperscript{27} demonstrated that TBX3 deficiency results in the expansion of atrial-type cells into the sinoatrial node domain.
and the partial loss of sinoatrial node-specific gene expression. Thus, TBX3 represses the atrial phenotype and regulates the development of the SAN.

1.a.iii. Electrophysiological properties of the sinoatrial node

The exact shape of action potential in the SAN varies among the different cell types located within the node. The smaller cells located in the center of the node elicits a slower action potential upstroke which reflects a lower expression of sodium channels and a depolarization phase that is dominated by the calcium inward current (see Figure 1)\textsuperscript{32}. SAN cells away from the center, located near periphery of the node, consist of larger cells which have a higher expression of sodium channels resulting in taller and faster action potentials (see Figure 1)\textsuperscript{33}. Peripheral nodal cells have an increased rate of diastolic depolarization which is due to the increase of the I\textsubscript{f} current in these cells\textsuperscript{34}. The increase in firing rate of the peripheral nodal cells occurs only in isolated cells, whereas in the intact heart, the increased firing rate is counteracted by the electrical coupling of these cells to the surrounding atrial tissue\textsuperscript{35}.

Automaticity in pacemaker cells requires voltage-dependent ion channels for the generation of pacemaking activity. Pacemaking activity can be considered as an oscillator that is generated by a time-varied outward current and an activated voltage-dependent calcium inward current occurring during the repolarization phase. There are three phases involved in the pacemaker action potential: the upstroke, phase 0; repolarization, phase 3; and spontaneous depolarization, phase 4.

The depolarization of the action potential, phase 4, occurs when the membrane potential is around -60 mV which opens the slow, inward depolarizing Na\textsuperscript{+} current also
known as the funny current ($I_f$)\textsuperscript{36}. As the membrane potential reaches about -50 mV, the T-type Ca\textsuperscript{2+} channel is opened to allow an inward Ca\textsuperscript{2+} current ($I_{Ca,T}$) to further depolarize the cell. As the depolarization approaches threshold at -40 mV the opening of the L-type calcium channel allows a slow inward current of Ca\textsuperscript{2+} ($I_{Ca,L}$). The additional influx of Ca\textsuperscript{2+} allows the cell to reach the action potential threshold for the upstroke of the action potential, phase 0. At this phase, the depolarization is primarily caused by the increase of Ca\textsuperscript{2+} from the L-type Ca\textsuperscript{2+} channel. As the membrane potential approaches the calcium equilibrium potential it results in a decrease in K\textsuperscript{+}\textsuperscript{36}. Here, during the upstroke phase of the action potential, the fast voltage-dependent K\textsuperscript{+} channels ($I_{Kr}$) are activated to drive the outward current which results in repolarization, phase 3. Simultaneously, the L-type-calcium channel is inactivated and closed and will reopen when the membrane potential reaches approximately -65 mV. The voltage-dependent K\textsuperscript{+} channels are deactivated slowly at the end of repolarization and during diastolic depolarization\textsuperscript{22}. Once the cell is completely repolarized at approximately -65 mV, the cycle will spontaneously repeat. A net inward current is required to overcome the outward components to initiate membrane depolarization which is generated by the association of the hyperpolarization activated current ($I_f$) with the sustained inward current ($I_a$), the T-type Ca\textsuperscript{2+} current ($I_{Ca,T}$), the L-type Ca\textsuperscript{2+} current ($I_{Ca,L}$) and the sodium current ($I_{Na}$)\textsuperscript{22}.

The $I_{Kr}$ current is the key repolarizing current in SAN cells where its inward rectification causes a decrease in membrane resistance during the repolarization phase and its deactivation results in a net current change during the early diastolic depolarization phase\textsuperscript{37}. It was observed that $I_{Kr}$ is dependent on extracellular K\textsuperscript{+} concentration in isolated pacemaker cells and may account for the sensitivity of the rate of pacing from these cells \textit{in vivo}\textsuperscript{37}. 
When $I_{Kr}$ is blocked by E-4031, a class II methanesulfonanilide compound, the automaticity of the cells is significantly slowed, but not completely terminated\footnote{38}. The lack of termination of the signal by E-4031 is explained by the presence of the electrotonic load on the SAN by the surrounding atrial tissue which has a more negative diastolic potential\footnote{22}. Thus, $I_{Kr}$ plays a role in action potential repolarization in SAN cells and also plays an important role in the coupling of SAN automaticity to the surrounding atrial tissue.

The $I_{f}$ current has been shown to play a key role in generating pacemaking activity in the SAN. $I_{f}$ is the only voltage dependent current expressed in the SAN where it is activated when the membrane is hyperpolarized. F-channels open during the late phase of repolarization near the maximum diastolic potential. $I_{f}$ initiates the first part of the diastolic depolarization until the activation threshold is reached for the T- and L-type channels\footnote{39};\footnote{40}. HCN4 is the predominant F-channel isoform that is expressed in the SAN. Thus, not surprisingly, embryos lacking HCN4 channels die in utero between day 9 and day 12 post coitus\footnote{28}. Younger embryos which are HCN4-deficient have almost complete $I_{f}$ suppression and cAMP regulation in the heart is eliminated.

The L-type Ca\textsuperscript{2+} channels ($I_{Ca,L}$) mainly contribute to the upstroke phase in the action potential of the pacemaker. It also plays a role in the generation of SAN diastolic depolarization\footnote{41}. The L-type Ca\textsuperscript{2+} channels are regulated by the phosphorylation state of the channel as it has been shown that these channels are regulated by PKA\footnote{42}.

The T-type Ca\textsuperscript{2+} channels ($I_{Ca,T}$) are expressed in the SAN, AVN and Purkinje fibers\footnote{43};\footnote{44};\footnote{45}. The pacemaking activity of $I_{Ca,T}$ has been illustrated in pharmacological and genetic data, but the precise description of how $I_{Ca,T}$ contributes to the diastolic depolarization is still unknown\footnote{22}.
The action potentials of primary pacemaking cells are characterized by a relatively slow upstroke, where the action potentials in the pacemaking cells are mainly driven by Ca$^{2+}$ and not Na$^{+}$\textsuperscript{22}. However, due to the two different distinctive cell types present in the SAN, small cells in the center and large cells in the periphery, two different I$_{Na}$ components have been functionally identified in the SAN— Tetrodotoxin (TTX)-sensitive I$_{Na}$ coded by Na$_v$1.1 and the TTX-resistant I$_{Na}$ coded by Na$_v$1.5\textsuperscript{46-50}. TTX-sensitive I$_{Na}$ mainly contributes to pacemaking in the adult mouse whereas the TTX-resistant I$_{Na}$ is involved in SAN intra-nodal conduction\textsuperscript{48-50}. When the TTX-sensitive I$_{Na}$ is blocked by TTX, the automaticity in the SAN has been shown to slow in the intact SAN as well as in isolated nodal cells\textsuperscript{48}. In action potential clamp experiments, the TTX-sensitive I$_{Na}$ is present during the late phase of diastolic depolarization as well as in the upstroke phase. The role of the Na$_v$1.5 (TTX-resistant I$_{Na}$) channel was illustrated in SAN pacemaking cells in Scn5a\textsuperscript{+/-} mice. In atrial-preparations from these mice, normal pacemaking in the center was observed but had a slower intra-nodal SAN conduction and exit block\textsuperscript{49}. Thus, it seems that Na$_v$1.5-mediated I$_{Na}$ does not participate in the generation of automaticity but contributes to the heart rate due to its involvement in contributing to the impulse propagation from the SAN to the atrium.

The role of I$_{St}$ in pacemaking is unknown due to the unidentified molecular basis of the st-channels. Shinagawa \textit{et al.}\textsuperscript{51} suggested that I$_{St}$ contributed to pacemaking by controlling the rate of diastolic depolarization. It was suggested that I$_{St}$ possibly contributed to pacemaking due to its low threshold of activation and slow inactivation rate indicating that I$_{St}$ is present throughout the pacemaker cycle.
Figure 1. The spontaneous action potential from two different cell types in the SAN. The small cells were observed in the centre of the SAN whereas the larger cells were located near the periphery of the node. The two different cell types elicit different patterns of action potentials. Reprinted from Shattock, 2006.\textsuperscript{32}
1.a.iv. Development of the sinoatrial node

During development, the sinus venosus is comprised of the following structures: the SAN, the venous side of the bilayered venous valves, and the right and left sinus horns. The earliest detected structure of the SAN was observed on embryonic day (E) 10.5 in the mouse. The cells forming the SAN arise from loose mesenchymal cells of the pericardial wall by E11.5 and the development of the SAN from here on occurs in the right sinus horn at the junction with the atrium. During the formation of the SAN, analyses have shown a molecular pattern that is characteristic of the formation of the SAN on the right side of the sinus venosus myocardium at E9.5 to E10 consisting of TBX3+/HCN4+/Cx40- cells. The initial TBX3+ expression in the sinus venosus results in the formation of the “tail” portion of the SAN extension along the right venous valve. The “head” structure of the SAN is a result of continual growth by proliferation and mesenchymal differentiation of the mesenchymal progenitors in the right sinus horn.

During development from E9 to E9.5, NKX2.5 is expressed in all myocardium except for the mesenchymal cells on the caudal-ventral-lateral side of the inflow tract. The non-expressing NKX2.5 cells express the T-box transcription factor TBX18. Interestingly, cells that differentiate after E9 to 9.5 in the sinus venosus and the SAN express TBX18 and not NKX2.5 which suggests that the sinus venosus and SAN myocardium differentiate from TBX18+/NKX2.5-negative mesenchymal precursors. Cell lineage studies have proved that the sinus venosus stems from noncardiac progenitors. In another study, TBX18 deficient mice were unable to form the large “head” component of the SAN which illustrates the importance of TBX18 for the recruitment of these mesenchymal precursors into the cardiac lineage. The initial formation of the sinus venosus and SAN is initiated between E9.5 and
E11 in the mouse where the subsequent growth to gain size occurs through slow proliferation of the cells\textsuperscript{53,54}.

TBX3 is the transcriptional repressor that is responsible for inhibiting prospective SAN cells from differentiating into working myocardium\textsuperscript{30,55}. TBX3 is regulated by the repressor genes SHOX2 and NKX2.5. In the mouse, TBX3 is selectively expressed in SAN primordium and arises in expression from E9.5-10. In Tbx3 deficient mice, an increase of working myocardium gene expression including Cx40, Cx43, Nppa, Scn5a was observed to be invading the SAN domain\textsuperscript{27}. Contrastingly, ectopic expression of Tbx3 in mice resulted in the activation of SAN genes including HCN4, Cx30.2 and the repression of the working myocardium genes. These findings implicate the undeniable importance of TBX3 in regulating the SAN phenotype.

Another key marker during SAN development is HCN4. HCN4, similar to TBX3, is regulated by the repressor pathway through Shox2 and NKX2.5 and is selectively expressed in the sinus venosus. HCN4 is initially expressed in the caudal part of the heart tube where it is NKX2.5\textsuperscript{+} at around E9.5\textsuperscript{28,29}. HCN4 expression is shortly down-regulated in this area and becomes activated in the newly differentiated myocardial cells in the sinus horn that are TBX18\textsuperscript{+} and NKX2.5-negative, which occurs between E9.5 and E14.5. Given that both HCN4 and TBX3 is activated in the NKX2.5-negative regions in the sinus venosus, it is suggested that NKX2.5 represses the expression of these genes, whereas NKX2.5 is required for the activation of the genes expressed in the atrial working myocardium (i.e. Cx40, Nppa). This was supported in studies by Mommersteeg et al.\textsuperscript{30} where NKX2.5 deficient embryos generated ectopic expression of HCN4 and TBX3 in the heart tubes.
Studies on SHOX2 in mice have indicated that it has a role as a negative upstream regulator of NKX2.5 in the sinus venosus during development\textsuperscript{56,57}. SHOX2 deficient mice exhibit an up-regulation of NKX2.5, Cx40 and Cx43 and a down-regulation of HCN4 and TBX3 in the region presumed to be the SAN primordium. This results in bradycardia and eventual loss of pacemaking activity in SHOX2 deficient mice. An additional gene involved molecularly in the development of the SAN is the T-box transcription factor gene TBX5 where it is expressed in a caudal-high gradient in the heart during development and is involved in the formation of early atria by regulating the expression of SHOX2 and TBX3\textsuperscript{58}. Tbx5 is expressed in all atrial and venous myocardium where its expression is regulated by retinoic acid signaling. TBX5 activates the expression of Shox2 and Tbx3 expression in the SAN region.

Another key gene involved in SAN formation is the transcription factor Pitx2 which controls the asymmetrical development of the heart\textsuperscript{59}. In Pitx2c-deficient mice, the formation of the SAN occurs at the right and left sinoatrial junction. Because the two SANs that are formed are indistinguishable, this suggests that Pitx2c is involved in the left-right heart formation by suppressing the formation of an SAN on the left sinus venosus\textsuperscript{30}.

1.b. Heterotrimeric G-proteins signaling

1.b.i. Overview

G-protein signaling pathways are ubiquitously present in eukaryotic organisms to play essential roles in cellular signal transduction by mediating a wide range of extracellular physiologic signals\textsuperscript{60,61}. G-protein coupled receptors and their associated heterotrimeric guanine nucleotide-binding regulatory proteins (G-proteins) transduce extracellular signals
across the plasma membrane to produce the appropriate intracellular response\textsuperscript{61}. In the basal state, the heterotrimeric G-proteins, the Gα subunit and the Gβγ heterodimer, are bound together as a quiescent complex\textsuperscript{62}. When a specific ligand binds to the G-protein coupled receptor (GPCR), the heterotrimeric G-protein complex becomes activated and can stimulate specific downstream effector pathways\textsuperscript{62}.

1.b.ii. G-protein coupled receptors and G-proteins

GPCRs are among the largest and most diverse family of proteins. The primary function of GPCRs is to transduce extracellular stimuli into intracellular signals. The diversity of GPCRs is due to the wide array of stimuli to which they must respond and the vast range of intracellular pathways they activate. The extracellular stimuli to which GPCRs respond to include, but are not limited to, neurotransmitters, light, odorants, lipids, proteins, amino acids, hormones, chemokines, and nucleotides\textsuperscript{63}. Due to the wide number of physiological roles played by GPCRs, they have been important therapeutic targets for treatment of many disease conditions. Not surprisingly, searches for therapeutic agents to act through GPCRs have been proven to be highly successful. Indeed, over 50% of all pharmaceutical drugs currently on the market target GPCRs\textsuperscript{64}.

All GPCRs are characterized by seven membrane-spanning helices with an extracellular amino-terminus and an intracellular carboxyl-terminus coupled to heterotrimeric G-proteins, Gαβγ\textsuperscript{61}. Ligand binding to the extracellular domain results in a conformational change in the receptor resulting in the activation of the heterotrimeric G-proteins. The activated GPCR catalyzes the exchange of guanosine diphosphate (GDP), bound to the α-subunit, for guanosine triphosphate (GTP). The Gα-GTP complex subsequently dissociates
from the receptor and the βγ-heterodimer to activate downstream effectors\textsuperscript{62}. There are four primary families of the Gα-protein: Gα\textsubscript{s}, Gα\textsubscript{i/o}, Gα\textsubscript{q/11} and Gα\textsubscript{12/13}\textsuperscript{65}. The different family of Gα proteins adds to the breadth of physiological responses that GPCRs encompass.

Termination of the signal occurs when the GTP bound to the α-subunit is hydrolysed to GDP resulting in the re-association and reformation of the heterotrimer Gαβγ and reassociation of the heterotrimer with the GPCR\textsuperscript{61}.

1.b.iii. Heterotrimeric G-protein signaling in the heart

Cardiovascular function is regulated by a variety of hormones and neurotransmitters, many of which are ligands for GPCRs\textsuperscript{66}. Given that over 200 cardiac GPCRs have been identified, drugs that target adrenergic and angiotensin GPCR signaling pathways constitute the majority of prescription medication for cardiovascular diseases\textsuperscript{65}. Adrenergic receptors, a class of GPCRs, are responsible for chemical signal transduction from the sympathetic nervous system to cardiovascular responses. In particular, the β-adrenergic receptor (βAR), β1AR is coupled to the Gα\textsubscript{s}-protein to regulate heart rate and contractility and the β2ARs are important for mediating smooth muscle relaxation and glycogenesis\textsuperscript{67,68}. The Gα\textsubscript{s} stimulates adenylyl cyclase (AC) resulting in the increase of intracellular production of cyclic AMP to modulate cardiac contractility via PKA binding\textsuperscript{65}.

GPCRs coupled to Gα\textsubscript{q} play an important role in cardiac growth, cardiac hypertrophy and myocyte apoptosis\textsuperscript{69}. For example, Angiotensin II stimulates the Gα\textsubscript{q}-coupled AT1R which results in vascular smooth muscle contraction\textsuperscript{70}. The angiotensin receptor blockers and angiotensin-converting enzyme inhibitors have been demonstrated to decrease cardiac hypertrophy, reverse cardiac remodeling, and delay the progression of heart failure acting via
the angiotensin II pathway\textsuperscript{65}. The $\alpha$-1-adrenergic receptors are involved in signals from the sympathetic nervous system which are also coupled to G\textsubscript{\alpha\_q} signaling\textsuperscript{71}. Activated $\alpha$-1-adrenergic receptors signal via the PLC\textsubscript{\beta}/DAG/PKC pathways to induce cardiac growth, cardiac and vascular smooth muscle cell contractile responses, and blood pressure\textsuperscript{65,71}. Another cardiac GPCR that is coupled to G\textsubscript{\alpha\_q} is the endothelin-1 receptor which promotes cardiac remodeling and hypertrophy\textsuperscript{72}.

G\textsubscript{\alpha\_i} inhibitory (G\textsubscript{\alpha\_i}) signaling opposes G\textsubscript{\alpha\_s} signaling by inhibiting AC and reducing the production of cAMP leading to the inhibition of contraction force in myocytes. Sustained G\textsubscript{\alpha\_i} stimulation can result in bradycardia and cardiomyopathy\textsuperscript{73}. In the heart, G\textsubscript{\alpha\_i} is coupled with M2 muscarinic receptors (M2Rs), $\alpha$-2-adrenergic receptors ($\alpha$2AR) and A1 adenosine receptors (A1Rs)\textsuperscript{65}. M2Rs coupled to G\textsubscript{\alpha\_i} mediates negative inotropic effects while the negative chronotropic effects are caused by I\textsubscript{K\textsubscript{Ach}} channel activation mediated by the G\textsubscript{\beta\gamma} subunit\textsuperscript{65}. In the presence of increased adrenergic drive, heart rate and contractability is regulated by the $\alpha$2AR coupled to G\textsubscript{\alpha\_i} which suppresses norepinephrine release from the synaptic nerves of healthy hearts to minimize the amounts of norepinephrine in the synaptic terminals\textsuperscript{74}. Activation of A1Rs is cardioprotective mediated by a reduction in heart rate, contractility, and attenuation of cardiovascular catecholamine stimulation\textsuperscript{75}.

1.c. Regulators of G-protein signaling

1.c.i. Overview

As mentioned above, GPCR activation leads to the exchange of GDP for GTP on the G\textsubscript{\alpha} subunit resulting in the dissociation of the heterotrimer into a GTP-G\textsubscript{\alpha} complex and a G\textsubscript{\beta\gamma} heterodimer. The extent and duration of GPCR signaling is determined by the balance
between the level of activation or amount of GDP-GTP exchange and the rate of GTP hydrolysis to GDP to deactivate of the signal.

The intrinsic rate of hydrolysis of GTP to GDP is known to be too slow to account for the rapid on-off kinetics needed to regulate rapid physiologic signaling events in the cardiovascular system. For example, the rate of GTP hydrolysis for the retinal G-protein transducin occurs on a time scale of several seconds in vitro whereas the termination of an activated living photoreceptor cell occurs in less than a second\textsuperscript{76}. The increased rate GTP hydrolysis in vivo, predicted the existence of a family of proteins that could increase the intrinsic rate of GTP hydrolysis by the $G\alpha$ subunits. This new family of GTPase activating proteins (GAPs) was aptly named the Regulators of G-protein Signaling (RGS) superfamily.

1.c.ii. Discovery of RGS proteins

The discovery of RGS proteins stemmed originally from studies carried out in yeast (\textit{Saccharomyces cerevisiae}) where the gene \textit{sst2} was identified as a negative regulator of $G\alpha$ in yeast pheromone response pathway\textsuperscript{77}. The \textit{sst2} mutants were hypersensitive to the pheromone signal and failed to desensitize following prolonged stimulation. Additional studies illustrated that a gain-of-function mutation to the \textit{sst2p} gene resulted in a loss of pheromone-stimulated gene transcription leading to cell cycle growth arrest\textsuperscript{78}. These findings lead to subsequent independent studies to identify other RGS proteins including GAIP (\textit{G$\alpha$}-interacting protein) found in the human with high affinity for $G\alpha_{13}$\textsuperscript{79}, G0S8 which is up-regulated in stimulated monocytes\textsuperscript{80}, and BL34/1R20 which is up-regulated by mitogenic stimuli\textsuperscript{81}. Through sequence alignment analysis, it was observed that there is a conserved block of $\sim$120 amino acid domain which contained the G-protein regulatory
sequences RGS superfamily resulting in G0S8 and BL34/1R20 being renamed to RGS2 and RGS1 respectively\(^80\).

1.c.iii. RGS proteins as GAPs

RGS proteins were first described to be GTPase activating proteins (GAP) for G\(\alpha\) subunits by Gilman and co-workers at the University of Texas Southwestern Medical Center in Dallas\(^82;83\). Several RGS proteins were shown to have a high affinity for the GDP/Mg\(^{2+}\)/AlF\(_4^-\)-bound G\(\alpha\) subunit, a situation that represents the transition state for GTP hydrolysis\(^82;83\). It was thus hypothesized that RGS proteins stabilize the transition state thereby lowering the activation energy barrier required for efficient hydrolysis of GTP\(^82\). RGS protein GAP function can increases the rate of intrinsic GTP hydrolysis on the G\(\alpha\) subunit by up to 1000 fold. Figure 2 shows the role of an RGS protein within the GTPase cycle. \textit{In vitro}, RGS proteins have been shown to interact with GTP-bound G\(\alpha_i\), G\(\alpha_q\) and G\(\alpha_{12/13}\), but not to G\(\alpha_s\)\(^83\).
**Figure 2.** The GTPase cycle. The inactive G-protein (left) is stable as a heterotrimer with a GDP molecule bound to the Gα subunit. Following activation by an activated GPCR associated to the heterotrimeric G-protein, the GDP is released in exchange for a GTP leading to the dissociation of the GTP-Gα complex from the Gβγ heterodimer (right). Each activated complex stimulates respective downstream effectors. Signaling through the downstream effectors continue until the GTP on the Gα-subunit hydrolyses to the GDP resulting in the reassociation of the Gα subunit with the Gβγ subunit, inactivating of G-protein signaling. However, this intrinsic GTPase activity is slow so this hydrolysis and deactivation is catalyzed by the action of the RGS family of proteins.
1.c.iv. RGS protein superfamily

The RGS protein superfamily is a large family consisting of over 30 members of highly diverse and multifunctional signaling proteins which bind to and activate the Gα-subunit\textsuperscript{84}. All members of the RGS superfamily share the highly conserved 120 amino acid long RGS domain. RGS proteins vary widely in size and structural domain enabling them to interact with different protein binding partners and have diverse cellular roles\textsuperscript{61}. Based on their structural and sequence homology, the RGS proteins have been classified into six distinct subfamilies\textsuperscript{85;86}. Four of these subfamilies display GAP activity which include the C/R7 subfamily (prototype RGS7), the D/R12 subfamily (prototype RGS12), the A/RZ subfamily (prototype RGSZ), and the B/R4 subfamily (prototype RGS4). The other two subfamilies, RGS-like subfamily (F/RL) and Axin-like subfamily (E/RA), have weakly homologous RGS domains and relatively poor Gα recognition and will therefore not be discussed.

1.c.iv.1. C/R7 subfamily

The C- or R7-subfamily consists of the RGS6, RGS7, RGS9 and RGS11 genes and their assorted splice variants\textsuperscript{77;85;87-89}. The main structural features unique to the C- or R7 subfamily are the DEP and GGL domains N-terminal to the RGS domain. The conserved DEP domain consists of ~80 amino acid sequence that is found in D\textsubscript{ishellved} (an intracellular part of the Wnt signaling pathway), E\textsubscript{GL}-10 (the first RGS protein discovered in \textit{C. elegans}) and P\textsubscript{leckstrin} (a major PKC substrate in platelets)\textsuperscript{90}. The DEP domain within the R7 RGS protein is thought to be for membrane localization\textsuperscript{91;92}. The role of the GGL domains in the R7 RGS proteins is to bind to a Gβ-subunit, Gβ5, displacing the requirement for a Gγ-subunit. Together, the R7 RGS-
Gβ5 has a role in modulating downstream effectors similar to the roles of the conventional Gβγ heterodimers\textsuperscript{90}.

1.c.iv.2. D/R12 subfamily

The D- or R12-subfamily consists of RGS10, RGS12, and RGS14 and acts as GAPs for the Gα\textsubscript{i}-family of Gα-subunits \textit{in vitro}\textsuperscript{85,86}. Similar to the R7 subfamily, these genes are alternatively spliced into numerous mRNAs to produce proteins that populate this subfamily. The R12 family members vary significantly in size and function with RGS10 consisting of 173 residues and RGS12 with 1447 residues. RGS10 belongs to the R12 family due to the sequence similarity of the RGS-domain, however it is more structurally similar to the B/R4 subfamily due to its lack of accessory modules. RGS12 and RGS14, however, share multi-domains C-terminal to the RGS-box that includes tandem RBDs (Ras-binding domains) and a GoLoco motif\textsuperscript{93}. The RBD allows the RGS protein to interact with small GTPases Rap1 and Rap2\textsuperscript{94}. The Go-loco motif allows for selective interaction with GDP-bound Gα\textsubscript{i}-family of Gα-subunits and prevents guanine nucleotide dissociation\textsuperscript{95,96}.

1.c.iv.3. A/Rz subfamily

The A/Rz subfamily consists of RGS17, RGS19, RGS20 and the founding member GAIP, the first mammalian RGS protein identified in yeast studies\textsuperscript{79}. Members of this family share a cysteine-rich cluster N-terminal to the RGS domain\textsuperscript{90}. This cysteine-rich region is thought to be heavily palmitoylated for strong membrane association\textsuperscript{97}. 
1.c.iv.4. B/R4 subfamily

The largest group of RGS proteins is the B/R4 subfamily including RGS1, RGS2, RGS3, RGS4, RGS5, RGS8, RGS13, RGS16 and RGS18. This family of RGS is comprised of the smallest proteins at 20-30 kDa in size where it contains an RGS-domain flanked by minimal N- and C-terminal domains. With no other recognizable motifs, it is suggested that their principal role is to regulate G-protein signaling through the RGS domain while the N-terminal amphipathic helix determines their subcellular localization. All members of the R4 subfamily, with one exception, have the ability to bind and regulate Gαi and Gαq signaling. The exception, RGS2, was shown to be selective for inhibition of Gq. It has also been suggested that the small amino terminal domains may play a role in targeting the Gα-GAP activity of these RGS proteins to specific receptors. For example, despite similar abilities of their RGS domains to regulate G-protein activity RGS1, RGS4 and RGS16 show large differences in their ability to inhibit muscarinic-, bombesin-, or cholecystokinin-receptor-promoted Ca²⁺ signaling in permeabilized pancreatic acinar cells.

1.c.v. RGS4

The focus of my thesis is RGS4 which belongs to the R4 subfamily and is the prototypical member of the R4 subfamily that selectively inhibits Gαi/o- and Gαq- but not Gαs- signaling. RGS4 was one of the first RGS proteins to be discovered, so its biochemical and cellular properties have been extensively studied, more so than any other RGS family member. The identification and discovery of RGS4 provided the first evidence of the functional significance of the sequence homology between the family of RGS proteins. Upon the discovery of the sequence homology, further database searches allowed the identification of
other putative RGS family members. RGS4 is found in both humans and rodents and is selectively expressed in the central nervous system and the heart\textsuperscript{106,107}.

1.c.v.1. RGS4 in the central nervous system

During embryonic development of the CNS, RGS4 is found to be expressed in the locus coeruleus, sympathetic ganglionic neurons, and cranial sensory and motor neurons\textsuperscript{108}. It was found that the expression pattern of RGS4 highly overlaps the expression pattern of acetylcholinesterase implicating a physiological role for RGS4 in the regulation of mACh receptor signaling\textsuperscript{107}.

A role for RGS4 in Parkinson`s disease was established in a study by Ding \textit{et al.}\textsuperscript{109} where high RGS4 expression was found in cholinergic interneurons of the striatum. In Parkinson`s disease, a loss in dopaminergic neurons in the striatum is also associated with an increase in acetylcholine release resulting in the exacerbation of motor symptoms of the disease. The loss of dopaminergic neurons also lead to the reduction of M\textsubscript{4}-muscarinic receptor mediated signaling attributed to the upregulation of RGS4\textsuperscript{110}.

In addition to Parkinson`s disease, RGS4 has been implicated as a potential etiological factor for schizophrenia. Post-mortem studies have shown a reduction of RGS4 expression in the frontal cortex of schizophrenics including the superior temporal gyrus\textsuperscript{111}. However, functional studies have yet to establish whether the levels of RGS4 expression are associated with the development of schizophrenia and the exact role of RGS4 in the etiology of this neurological disorder is unclear.
1.c.v.2. RGS4 expression in the SAN and heart

The autonomic nervous system regulates heart rate via specialized autorhythmic cells located in the sinoatrial node of the heart\textsuperscript{112}. Heart rate is controlled by the competing activities of the sympathetic and parasympathetic nervous system. The stimulation of the sympathetic $\beta$-adrenergic receptors accelerates the heart rate, while the stimulation of the parasympathetic muscarinic receptors decreases heart rate. Sympathetic neurotransmitters increase adenylyl cyclase activity, intracellular cAMP concentrations and protein kinase A through the activation of $G_{\alpha_s}$-coupled $\beta$-adrenergic receptor. Consequently, the increased activity of HCN channels and voltage-gated $Ca^{2+}$ channels, whose activity are increased by upregulated levels of cAMP, result in a greater firing rate of pacemaking cells\textsuperscript{40,113}. In contrast, parasympathetic activity decreases the heart rate through the activation of $G_{\alpha_{i/o}}$-coupled cholinergic M\textsubscript{2} muscarinic receptors (M\textsubscript{2}Rs)\textsuperscript{114}. The reduction in heart rate by parasympathetic activity is mediated by both $G_{\alpha_{i/o}}$-subunits and the $G_{\beta\gamma}$-heterodimer. $G_{\alpha_{i/o}}$ activation results in changes in phosphodiesterase activity and inhibition of adenylyl cyclase activity resulting in the decrease in intracellular cAMP concentrations and protein kinase A activity which ultimately leads to the decrease in depolarizing currents from HCN and L-type $Ca^{2+}$ channels\textsuperscript{113-115}. The $G_{\beta\gamma}$-heterodimer directly activates the G protein-coupled inward rectifying potassium channel (GIRK; composed of a tetramer made from Kir3.1/Kir3.4 subunits) causing membrane hyperpolarization.

Our lab has demonstrated that RGS4 is highly expressed in the sinoatrial node (SAN) of the mouse\textsuperscript{116}. RGS4 is known to attenuate $G_{\alpha_{i/o}}$ but not $G_{\alpha_s}$ activity. Thus, as expected, hearts from RGS4 knockout mice showed dramatically increased sensitivity to parasympathetic activity. Specifically, RGS4 knockout mice showed enhanced bradycardic responses to
carbachol treatment *in vivo* that was associated with increased levels of $I_{K,Ach}$ (the potassium current carried by the GIRK channel in response to M2R Gi activation) in isolated SAN myocytes. Furthermore there was an increase in the maximal negative diastolic potential in the presence of carbachol and loss of proper GIRK channel desensitization. This suggests that RGS4 may be specifically involved in a feedback mechanism regulating $M_2R$-$G_{i/o}$-GIRK complexes as was suggested by Doupnik *et al.* 87. Taken together these data suggest that reduction of RGS4 expression in the SAN could increase susceptibility to bradycardia and arrhythmia.

1.d. Stem cells

1.d.i. Embryonic stem cells

Embryonic stem cells (ESCs) are pluripotent progenitor cells that have the potential to generate any of the cell types present in the body. Many conditions are thought to be due to the lack of one or more crucial cell population that cannot be replaced by the body including, but not limited to, heart failure, stroke, diabetes, neurodegenerative disorders, blindness, spinal cord injury, osteoarthritis and kidney failure 8. In addition, stem cells provide a means for regenerative medicine since ESCs generate clinically relevant numbers of cells of a specific cell population for tissue repair and regeneration. The use of ESCs also provides a potential tool to evade the dangers of tissue rejection following transplantation via the generation of patient-specific ESCs that can be used directly for transplant therapy or for targeted drug screening protocols. However, despite their promising potential, the same plasticity that gives ESCs the potential to become numerous different cell types can also makes them very difficult to control.
Pluripotent ESCs are derived at the blastocyst stage from the inner cell mass\textsuperscript{117,118}. The pluripotent nature of ESCs was demonstrated in a study by Bradley \textit{et al.}\textsuperscript{119} where injection of ESCs into the host blastocytes showed contributions of these cells to all tissues in adult mice. Importantly, once derived, these ESCs can be maintained and expanded as undifferentiated cells indefinitely and can retain normal karyotypes following extended passages in culture. ESCs are usually maintained in co-culture with mouse embryonic feeder cells\textsuperscript{117,118}. The key component in maintaining these cells undifferentiated in culture is leukemia inhibitory factor (LIF)\textsuperscript{120,121}. In the presence of recombinant LIF and appropriate batches of fetal calf serum (FCS), it is possible to maintain the growth of undifferentiated ESCs without the need for murine embryonic fibroblasts (feeder cells)\textsuperscript{120,121}. However, the use of FCS in cultures is not ideal due to the poorly defined combination of factors in the serum. Ying \textit{et al.}\textsuperscript{122} discovered the role of BMP4 in ESC growth such that in the presence of LIF can suppress differentiation of ESCs and replace the FCS supplement. Thus, it is possible to robustly expand ESCs in the absence of serum and feeder cells. Recently, Gertsenstein \textit{et al.}\textsuperscript{123} showed that inhibiting the WNT and ERK signaling pathways significantly improve the derivation of the cell line and their differentiation potential. Through the use of the two inhibitors, 2i: MEK inhibitor PD0325901 and GSK3 inhibitor CHIR99021; a more stable karyotype analysis was achieved. The use of 2i media improved cell lines and can be used to expand ESCs in culture without the use of feeder cells.

1.d.ii. Cardiovascular differentiation from mouse embryonic stem cells

When the factors that maintain ESCs as stem cells are removed, the ESCs will differentiate. Under proper conditions, the ESCs will generate cells that consist of the three embryonic germ layers: mesoderm, endoderm and ectoderm\textsuperscript{124,125}. Since these cells were
derived from the inner cell mass, it is not surprising that the ESCs do not differentiate to trophectoderm in culture. Differentiation of ESCs follows one of the three basic methods that have been developed: (a) culturing ESCs directly on stromal layers, (b) culturing ESCs as monolayers, or (c) formation of embryoid bodies (EBs) as three-dimensional aggregates\textsuperscript{124,126-128}. It is debated as to which approach is the best for differentiation as each method has the ability to differentiate the ESCs into a broad spectrum of cells in culture with its advantages and disadvantages. Culturing these cells on stromal layers provide beneficial growth by promoting effects of the particular cell line but the undefined factors that are produced by the stromal cells may influence the differentiation of the ESCs to different undesired cell types. In addition, it may be difficult to separate the stromal cells from the differentiated cells. Differentiation of ESCs as EBs increases the cell-cell interactions as they form three-dimensional structures which may be important for certain developmental programs. However, the complexity of EBs are a disadvantage such that the production of cytokines and inducing factors inside these structures can complicate the interpretation of experiments when trying to understand lineage commitment from certain signaling pathways. Finally, differentiation of ESCs as monolayers can minimize the complexities of three dimensional structures and the effects of stromal cells. However, the choice of extracellular matrix proteins to underlie the ESCs is crucial as different proteins can affect the survival of different ESCs.

Subpopulations induced from the mesoderm include hematopoietic, vascular, cardiac and skeletal muscle lineages (Figure 3). As previously discussed, with cardiomyocytes emerging from the mesoderm cell population, it is of importance to understand the mechanisms that regulate mesoderm induction. Kattman \textit{et al.}\textsuperscript{129} recently identified markers that are used to evaluate the efficiency of the cardiac mesoderm induction for eventual cardiovascular
development. The upregulation of the VEGF receptor-2 (Flk-1 or KDR) and PDGF receptor-α (PDGFR-α) are receptors that are broadly expressed on the cells of this subpopulation. The key signaling pathway involved in optimizing this germ layer induction are activin and BMP4 where different cell lines require different concentrations of Activin A and BMP4 to generate optimal expressions of Flk-1/KDR+/PDGFR-α+. In addition, varying concentrations of activin/Nodal and BMP signaling and the upregulation of Flk-1/KDR lead to a mesoderm fate of various lineages (Figure 3). The activation of the activin/Nodal and BMP pathways to generate cardiac mesoderm differentiation are consistent with their physiological activities occurring in vivo.

During embryonic development, agonists and antagonists of these pathways forms a gradient of signaling across the developing embryo to generate the specific germ layer populations. Gastrulation and induction of primitive streak populations require a distal-proximal Nodal gradient. Low levels of Nodal signaling promote the development of the posterior mesoderm and increasing levels of Nodal signaling promote more anterior populations including cardiac mesoderm and definitive endoderm (Figure 3)\textsuperscript{10,130-132}. BMP4 signaling has a dominant posteriorizing effect. In human pluripotent stem cells, signaling pathways may require inhibition with the addition of pathway antagonists in a stage specific manner to promote cardiomyocyte development which reflects the variations in cell lines with respect to the endogenous levels of factors produced. Furthermore, the differentiation kinetics of different cell lines may require evaluation to determine the optimal stage of germ line induction. Another key pathway for early differentiation is the Wnt signaling pathway. Although Wnt signaling is essential for mesoderm induction, its subsequent inhibition is crucial for promotion and specification for precardiac mesoderm differentiation.
The study by Kattman et al.\textsuperscript{129} illustrated that higher concentrations of activin/nodal was required for the generation of a Flk-1/KDR\textsuperscript{+}/PDGFR-\(\alpha\)\textsuperscript{+} cardiac mesoderm cell populations in comparison to the generation of Flk-1/KDR\textsuperscript{+}/PDGFR-\(\alpha\)\textsuperscript{−} hematopoetic/posterior mesoderm, which is consistent with the gradient effect of nodal observed in the embryo. In addition, increasing concentrations of BMP4 resulted in the loss of cardiac mesoderm cell populations and increased in the Flk-1/KDR\textsuperscript{+}/PDGFR-\(\alpha\)\textsuperscript{−} hematopoetic/posterior mesoderm cell population which demonstrates the posteriorizing effect of BMP signaling. This shows that the stem cell models used to generate various cell populations recapitulates what is observed in embryonic development.

As described, it has been illustrated by Kattman et al.\textsuperscript{129} that through the optimization of cardiac mesoderm development it is possible to generate populations that are highly enriched in cardiomyocytes. However, using the receptors Flk-1/KDR and PDGFR-\(\alpha\), as described earlier, as a marker of cardiac mesoderm, their expression does not always correlate to efficient differentiation of cardiomyocytes. It is still unclear whether the cell populations that are Flk-1/KDR\textsuperscript{+}/PDGFR-\(\alpha\)\textsuperscript{+} represent cardiac mesoderm or whether they mark their potential to be cardiac, since their absence indicates the lack of cardiac potential.

1.e. Rationale

It is known that RGS4 potently inhibits the \(\text{G}_\alpha_{\text{i/o}}\) protein coupled to cholinergic M2 muscarinic receptors (M\(_2\)R). Parasympathetic activity results in decreased heart rate cause by membrane hyperpolarization via agonistic ligand binding to the M\(_2\)R\textsuperscript{133}. Subsequently, G protein dissociation allows G\(\beta\gamma\) to activate the G protein-coupled inward rectifying potassium (GIRK) channels\textsuperscript{133}. The inhibition of \(\text{G}_\alpha_{\text{i/o}}\) by the RGS4 results in accelerated deactivation of
the GIRK channel. Thus, our lab has demonstrated that RGS4 is an important regulator for parasympathetic signaling at the SAN and consequently making it a potential clinical target for treatment and prevention of heart diseases.

It has also been shown that the RGS4 mRNA is highly expressed in sinoatrial node myocytes (SAN) relative to its expression in the atria and ventricles\textsuperscript{116}. Cifelli \textit{et al.} showed that RGS4 expression in the SAN is 4-fold higher the atria and 16-fold higher than the ventricles\textsuperscript{116}. Thus, RGS4 expression may be useful marker for the identification of SAN myocytes relative to other cardiac and non-cardiac cell types. All of the currently available data for RGS4 expression in the heart comes from adult tissues. Accordingly, the timing of RGS4 upregulation in SAN cells in relation to developmental cues, parasympathetic innervation, and initiation of the heart beat during cardiac development are unknown. We thus propose to further our understanding of SAN development by studying RGS4 expression during the specification of different myogenic lineages. Moreover, given the relatively high level of expression for RGS4 in the SAN we propose that RGS4 expression may be useful tool for increasing the efficiency of SAN myocyte identification and isolation during cardiac cell progenitor differentiation from pluripotent mESCs.
Figure 3. Embryonic stem cell differentiation. A scheme illustrating the factors involved in differentiation of ESCs into ectoderm, mesoderm and endoderm cell populations. The sensitivity of the factors are illustrated that the three main pathways involved are the BMP, activin/Nodal and Wnt signaling pathways. A gradient of expression of these factors generate different cell populations during development. High concentrations of BMP4 induces the posterior populations resulting in mesoderm and skin cells. A gradient of activin/Nodal signaling generates various cells from vascular to hematopoietic to cardiac to skeletal cells whereas high levels of activin/Nodal signaling results in definitive endoderm cell populations. Reprinted from Keller, 2005\textsuperscript{134}.
1.f. Aims and Hypothesis

Hypothesis 1

The hypothesis of this study is that during development RGS4 expressing cells will arise from the mesoderm cell population. Here, we plan to characterize changes in cardiac RGS4 expression in mice through embryonic stage to young adulthood and in mESCs that are subjected to cardiac cell differentiation.

Hypothesis 2

I hypothesized that selection of RGS4 expressing cells during cardiac differentiation will lead to a more efficient production and selection of autorhythmic SAN-like cells at the level of their gene expression profiles and electrophysiologic characteristics. We would like to determine whether RGS4 is a useful tool as a marker for increasing the efficiency of generating SAN-like myocytes during the cardiac cell differentiation. This will be achieved by sorting for RGS4-expressing cells during differentiation and determination of their physiologic characteristics relative to non-expressing cells.
Chapter 2

Materials and Methods

2.a. Animals

All experiments conformed to the Guide for the Care and Use of Laboratory animals Published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996), Institutional Guidelines and the Canadian Council on Animal Care\textsuperscript{135}.

2.a.i. RGS4 reporter-knockout mouse—RGS4-LacZ

The \textit{Rgs4}\textsuperscript{tm1Dgen/J} mouse strain backcrossed >six generations into a C57Bl/6 background was obtained from Jackson Laboratory (Bar Harbor, Maine, http://jaxmice.jax.org/strain/005833.html). An IRES-LacZ-neomycin cassette was inserted into the first intron to delete 58 base pair of coding sequence and the entire first intron (Figure 4). Mice were genotyped following the protocol provided by Jackson Laboratory.

To study the RGS4 expression pattern during development, RGS4 null male mice were mated with wild type female mice. The female mice were checked daily in the morning for sperm plugs. Detection of sperm plug indicated Day E0.5 pregnant. Mice were subsequently sacrificed by cervical dislocation at selected time points for embryo extraction. Given the size of the embryo, the embryonic heart was extracted if the heart was large enough. The isolated embryos or isolated embryonic hearts were stained for LacZ (staining procedure discussed in 2.b).
**FIGURE 4**

![Schematic diagram of RGS4 gene modification](image)

Figure 4. Schematic representing LacZ-Neo cassette insert into RGS4 gene. An IRES LacZ-Neo cassette was inserted to delete the entire first intron and 58 bp of coding sequence (red) which disrupts the translation of the functional RGS4 gene. This strategy allows the transcription of the insert to be driven by the endogenous promoter of RGS4, *in vivo*. 
2.a.ii. RGS4 reporter-transgenic mouse—RGS4-GFP

Homozygous RGS4 mES cells were derived from these mice by the Toronto Centre for Phenogenomics (Toronto, Ontario) using the 2i protocol\textsuperscript{123}. Mice were genotyped following the protocol provided by Jackson Laboratory.

2.a.iii. Genomic DNA isolation to genotype mice

Cell or tissue samples were digested with a lysis buffer containing 100 mM Tris-HCl pH 8.5, 5 mM EDTA, 0.2% SDS and 200 mM NaCl supplemented with 1 mg/ml proteinase K in a 55°C waterbath overnight. The digested sample was washed with once phenol:chloroform:isoamyl alcohol (25:24:1) (Sigma-Aldrich) followed by a wash with Chloroform (EMD). The DNA was precipitated using 30 µl of 3 M sodium acetate pH 5.2 and 750 µl of 100% ethanol (Commercial Alcohols). The DNA was recovered by centrifugation at 14'000 rpm for 10 minutes. A final purification of the DNA consisted of a wash with 70% ethanol. The DNA was air-dried and re-dissolved in an appropriate volume of distilled water based on DNA pellet size and incubated in a 37°C waterbath for 5 minutes.

2.b. Analysis of β-galactosidase gene expression

For detection of reporter gene expression in isolated embryos or cardiomyocyte monolayers, the embryos or isolated hearts were excised and rinsed in PBS with MgCl\textsubscript{2} and subsequently fixed in 2% paraformaldehyde (PFA) at room temperature for 15 minutes. Following fixation, the tissue was rinsed several times with PBS with MgCl\textsubscript{2}. For precipitation of LacZ, tissues were submerged in PBS containing 0.02% Nonidet P-40, 0.01% SDS, 2 mM MgCl\textsubscript{2}, 5 mM K\textsubscript{3}Fe(CN)\textsubscript{6}·3H\textsubscript{2}O, 5 mM K\textsubscript{4}Fe(CN)\textsubscript{6} and 1mg/ml 4-chloro-5-bromo-3-indolyl-β-
galactopyranoside (X-gal) overnight at 37°C. Stained tissues rinsed with PBS with Mg\(^{2+}\) and were further fixed at room temperature in 4% PFA at room temperature for 15 minutes. The tissue was subsequently rinsed several times with PBS with Mg\(^{2+}\). The tissue was stored for further analysis in PBS+Mg\(^{2+}\) at 4°C. Images of the excised tissue were collected using a PixeLINK® camera.

2.c. Cryosectioning of embryo/mouse tissues

Samples were fixed in 4% PFA for 30 minutes at room temperature and equilibrated with 30% sucrose (weight/volume) overnight at 4°C. Samples were immersed in OCT compound before freezing in dry ice cooled 2-methylbutane. Cryosections (8-10 micron) were mounted on a surface-treated glass slide and stained for LacZ (as described above). Stained sections were counterstained with Nuclear Fast Red (Vector Laboratories), dehydrated (series of 70%, 90% and 100% ethanol followed by 100% Xylene), mounted with Cytoseal (Fisherbrand) and photographed.

2.d. Immunohistochemistry

Cryosectioned embryos were stained with an HCN4 antibody to determine that RGS4 is highly expressed in cells representing the sinus region. The sections were post-fixed in 10% formalin, permeabilized with 0.1% Triton-X 100 (Bioshop) and blocked for 1 hour with 10% FBS. The sections were then incubated with rabbit anti-HCN4 IgG raised against residues 119-155 of human HCN4 (1:100 dilutions; Alomone Labs) in 1% BSA at 4°C overnight. The monolayer was subsequently incubated for 40 minutes at room temperature with a horseradish peroxidase-coupled goat anti-rabbit IgG secondary antibody (1:500 dilution in 1% BSA; Vector
laboratories). The monolayers were then stained by an immunoperoxidase kit (Vectastain, Avidin: Biotinylated enzyme complex kit PK-4000, Vector NovaRED peroxidase substrate, SK-4800, Vector Laboratories) according to manufacturer’s instruction. Stained sections were counterstained with Hematoxylin (Sigma-Aldrich), dehydrated (series of 70%, 90% and 100% ethanol followed by 100% Xylene), mounted with Cytoseal (Fisherbrand) and photographed. The adjacent sections of LacZ and HCN4 were compared.

2.e. Mouse embryonic stem cell culture

The mESCs were maintained and expanded in 0.1% gelatin coated 6-well dishes on irradiated mouse embryonic fibroblast (MEF) feeder cells with a serum-free (SF) embryonic stem cell (ES) media supplemented with 1% leukemia inhibitory factor (LIF) 2i inhibitors, 1 μM PD0325901 (Stemgent) and 3 μM CHIR99021 (Stemgent). Cells are frozen and stored in liquid nitrogen in Iscove’s modified Dulbecco’s medium (IMDM) with 40% FCS and 10% dimethyl sulfoxide (DMSO) (Sigma-Aldrich).

2.e.i. mESC differentiation into cardiomyocytes

Prior to the onset of differentiation cells are required to be feeder depleted. Thus, cells were passaged twice on 0.1% gelatin-coated plates in serum-free ES media (SFES) supplemented with 1% leukemia inhibitory factor and 2i inhibitors to deplete the mESC of feeder cells.

All cell lines used were differentiated in SF media as previously described by Kattman et al. and pictorially described in Figure 5. In brief, to initiate the differentiation embryoid bodies were generated from mESCs. Feeder depleted mESCs were trypsinized with TrypLE
(Invitrogen) and plated at a density of 100,000 cells/ml in SF media without any growth factors for 48 hours. At Day 2, depending on the cell line, the EBs were either kept intact or dissociated and the cells were reaggregated in SF media supplemented with varied concentrations of human Activin A (R&D systems) and BMP4(R&D systems), and 5 ng/ml VEGF(R&D systems). For cytokine grid experiments, aggregation induction was carried out in 24well low-cluster dishes (Corning, Corning, NY) in a total volume of 250 μl. For all other mESC experiments, the cells were differentiated in 10 cm petri grade dishes (Bectin Dickenson). At Day3+15hs or 39 hours later, EBs were harvested and analyzed by FACS or used for cell sorting.

Sorted cells or unfractionated populations were cultured in cardiac conditions as described in Kattman et al. (2006). or 6 x 10^4 – 10 x 10^4 cells were seeded into individual wells of a96-well flat bottom plate (Becton Dickenson, Franklin Lakes, NJ) coated with 0.1% gelatin cultured in StemPro-34 SF medium (Invitrogen) supplemented with 20 μM ascorbic acid (Sigma), 2mM L-glutamine, 150 ng/ml hDkk, 10 ng/ml bFGF, 5 ng/ml VEGF, and 300nM SB (R&D Systems). Cells were maintained with StemPro-34 SF medium supplemented with 20 μM ascorbic acid, 2 mM L-glutamine, 10 ng/ml bFGF and 5 ng/ml VEGF, for 4 or 5 days. Depending on the cell line, intact EBs were washed with IMDM and replated. Cells were harvested and analyzed by flow cytometry on D9.0. Beating monolayers or EBs were imaged and videos were taken on the Leica camera.
**Figure 5.** An outline of the protocol used for the differentiation of mESCs to the cardiac lineage. mES cells previously expanded on feeder cells are feeder depleted on 0.1% gelatin coated plate twice prior to induction. Day 0—cells are placed in 10cm dishes in a primary culture with serum-free media supplemented with ascorbic acid and MTG. Day 2—serum-free embryoid body induction—serum-free media is supplemented with VEGF, and the empirically determined concentrations of Activin A, BMP4 and Wnt3a. Day 3—cells are screened for double positives expressing PDGFR and Flk-1, and are plated for cardiomyocyte monolayer formation or kept as embryoid bodies. Day 7—spontaneously beating cardiomyocytes are observed.
2.f. FACS analysis and cell sorting

D3.5 EBs generated from ESC differentiation were dissociated with TrypLE (Invitrogen) into single cell suspension. Cells were incubated with anti-mouse Flk1-biotin for 30 minutes on ice. The cells were then washed twice with PBS and 5% FCS and incubated with anti-mouse PdgfR-α (CD140A)-phycoerythrin (PE) (clone ABA5, eBioscience, San Diego, CA) and APC-streptavidin (BD Pharmingen) covered from light for 30 minutes on ice. Following two additional washes with PBS/5%FCS, cells were analyzed on an LSRII flow cytometer (Becton Dickson). FACS data were analyzed with FlowJo software (Treestar, Ashland, OR).

D9.0 EBs or cardiac monolayers differentiated from mouse ESC was collagenized for 15-30 minutes at 37°C. Cells were subsequently incubated with trypsin for 3 minutes at 37°C for dissociation into single cell suspension. Cells were either stained with DAPI and sorted for DAPI negative and GFP positive cells and/or were fixed with 4% PFA on ice for 20 minutes for cardiac troponin T analysis by FACS. After fixing, cells were washed twice with FACS buffer (PBS with 5% FCS) then permeabilized with two washes using FACS buffer + 0.5% Saponin. The cells were incubated with troponin T Ab-1 (Neomarkers) for 30 minutes or overnight at 4°C. Cells were incubated for 30 mins on ice with goat anti-mouse Ig allophycocyanin (APC) (Becton Dickson). Cells were analyzed on LSRII flow cytometer (Becton Dickson). FACS data were analyzed with FlowJo software (Treestar, Ashland, OR).

2.g. RNA isolation and RNA analysis

To measure protein expression during the differentiation process, total RNA was prepared using the RNAqueous Micro Kit (AMbion) following company protocol. All samples were treated with DNase I to remove all genomic DNA from the sample. RNA (100 ng-1 μg)
was reverse transcribed into cDNA with oligo (dT) and random hexamers primers using Superscript™ II RT kit (Invitrogen) following manufacturers protocols. cDNA was diluted to a final concentration of 1 ng/μl. Two microliters of the cDNA mixture was used as a template for real-time quantitative (q) PCR on a MasterCycler RealPlex (Eppendorf) using SYBR Green JumpStart ReadyMix (Sigma). A 10-fold dilution series of sonicated mouse genomic DNA standards ranging from 50 pg/ml to 50 ng/ml was used to evaluate the efficiency of the PCR and to calculate the copy number of each gene relative to the housekeeping gene Actb. Refer to Table 1 for a list of all qPCR primers used.

2.h. Statistical methods

Unless otherwise stated, data are shown as mean values ± standard error of the mean (SEM). Data were analyzed using one-way ANOVA and unpaired student’s t-tests. To determine the significance, results with p-value of less than 0.05 (p < 0.05) was deemed significant. All data was analyzed using Microsoft Excel.
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**Table 1:** PCR primers used for mRNA quantification
Chapter 3

Results

3.a. Animal Study

3.a.i. Embryonic heart staining – whole mount

To determine the onset of RGS4 expression during murine cardiac development, embryos were isolated starting at 9.5 days post coitus (E9.5) with increasing time points. E9.5 was selected as the starting time point to collect embryos due to previous literature (as discussed in the Introduction) citing that the expression of HCN4, the key marker for SAN tissue, arises at around E9.5-E10 during development. Due to the small size and fragile nature of cardiac tissue in developing mouse embryos, intact hearts were successfully isolated from embryos at day E14.5 embryos and older. Whole-mount intact embryonic heart showed intense RGS4-LacZ expression in a crescent-shaped pattern within the right atrium from E14.5 embryos and onwards (Figure 6B). Earlier time points to determine the onset of RGS4 expression during development were studied by collecting the embryos and sectioning them as discussed in the following section (Section 3.a.ii).

3.a.ii. Immunohistochemistry and cryosectioning of embryos

To determine the onset of RGS4 expression in murine heart development, mouse embryos were collected from E9.5 to E14.5 for cryosectioning and the sections were stained for RGS4-LacZ. To determine whether RGS4 expression in early heart development was located in the SA node region, parallel cross sections were stained by immunohistochemistry for the SAN marker protein HCN4. RGS4-LacZ expression was not observed in the SAN region at
timepoints E10.5 and earlier (Figure 6A). Its expression was first noted in E11.5 as indicated by the colocalization of RGS4-LacZ expression with HCN4 expression in parallel cross sections of the embryos (Figure 6A). Once RGS4-LacZ expression was observed in the SAN region, all subsequent time points showed a similar staining pattern indicating that this gene continues to be expressed selectively in the SAN region throughout development and cardiac maturation post-natal (Figure 6B).
FIGURE 6

**Figure 6.** In vivo expression of RGS4 during murine heart development. (A) Parallel cross sections of the SAN region stained for LacZ expression (blue staining) and labeled by immunohistochemistry for the SAN-specific antigen HCN4 (red staining). E10.5 and E11.5 are shown at 10x. Black area indicates region of the SAN as stained positive for HCN4 (B) Whole-mount intact embryo hearts collected from E14.5 onward consistently show high and selective RGS4-LacZ expression in the SAN region as indicated by the yellow arrow. Scale bars = 1 mm.
3.b. Stem cells: RGS4-LacZ line

3.b.i. Determination of Activin A and BMP4 concentrations

For a serum-free differentiation of the mouse ES cells (mESCs), embryoid bodies were generated in culture for 2 days in serum free medium in the absence of any factors (see Figure 5 for the protocol). At day 2 the EBs are harvested where the cells are dissociated and reaggregated in serum-free media supplemented with VEGF and varying concentrations of BMP4 (2-16 ng/ml) and Activin A (0.01-10 ng/ml) in a grid format. Activin A and BMP4 are used at the primitive streak and mesoderm induction stage of differentiation, which are crucial for cells to differentiate into cardiomyocytes. The EBs were then analyzed by FACS 27 hours later (Day 3 + 3h) to determine the optimal ratios of Activin A and BMP4 to yield the highest percentage of Flk-1+/PDGFR⁺ (see Figure 7). In low cytokine concentrations, ESCs generate three different cell populations Flk-1⁻/PDGFR⁻, Flk-1⁻/PDGFR⁺, and Flk-1⁺/PDGFR⁻. With increasing concentrations of Activin A in the presence of low concentrations of BMP4, the expressions of Flk-1⁺/PDGFR⁺ cell populations increased to almost 60%. High levels of BMP4 (10 ng/ml) yielded some of the highest levels of Flk-1⁺/PDGFR⁺, but the use of higher concentrations of BMP4 are usually avoided as they tend to promote the differentiation of other undesired cell types. Thus, the concentration for Activin A and BMP4 selected as optimal for the RGS4-LacZ mESC line for differentiation into cardiomyocytes are 8 ng/ml and 1 ng/ml, respectively.
Figure 7. RGS4-LacZ mESC Activin A and BMP4 optimization grid. Optimal ratios of Activin A and BMP4 used for the induction of mouse embryonic stem cells (mES) were determined empirically to give the highest percentage of cardiac troponin T cells (cTNT) by measuring for double positive cells expressing Flk-1 and PDGFR. Y-axis = Flk-1, X-axis = PDGFR. Upper right quadrant indicates double positive cells expressing both Flk-1 and PDGFR. Further testing indicates that 1 ng/ml of BMP4 and 8 ng/ml of Activin A provides the best results. While higher concentrations of Activin A seem to provide more double positives, uses of higher concentrations are not well-tolerated in the cells later in the differentiation process.
3.b.ii. Effects of SB-431542 on differentiation

As shown by Kattman et al. (2010), the inhibition of the activin/Nodal pathway after the development of a cardiac mesoderm population increases the size of cardiomyocytes as measured by the cardiac isoform troponin T (cTNT). The increase was observed when the activin/Nodal pathway is inhibited by SB-431542 (hereafter referred to as SB) which suggests that prolonged nodal signaling actually inhibits cardiac specification. Supplementation of SB to the cultures resulted in an increase in the frequency and total number of cTNT+ population in cultures from 1.37% cTNT+ population in –SB day 3 media (Figure 8C) to 9.80% in +SB day 3 media (Figure 8B). As shown to be beneficial to cardiac differentiation, subsequent experiments for cardiac differentiation were supplemented with SB.

**FIGURE 8**

![Figure 8](image)

**Figure 8.** The effects of SB-431542 on differentiation. Efficiency of the use of SB-431542 was measured as the efficiency to increase the number of cardiomyocytes as measured with cardiac troponin on day 10 of the differentiation. (A) indicates 0.27% of cardiomyocytes differentiated in media supplemented with SB and BMP4. (B) indicates 9.8% of cardiomyocytes differentiated in media supplemented with SB only. (C) indicates 1.37% of cardiomyocytes differentiated with no supplementation of SB or BMP4 to the media/
3.b.iii. RGS4 expression in cardiomyocytes from differentiated mESCs

At Day 3 + 3h of the differentiation process, cells are analyzed or sorted for Flk-1⁺/PDGFR⁺ and plated as monolayer for the induction of the cardiac mesoderm and expansion of the cardiovascular lineage. During this process, the morphology of the cells changes daily (see figure 10) and undergoes a “pillowing effect” (see Figure 9a). In cultures where pacemaker cells are present the cardiomyocytes begin to spontaneously contract at day 7 and continue to contract indefinitely. In order to visualize expression of RGS4 in the cardiomyocytes the monolayers are fixed and incubated with β-galactosidase substrate to mark LacZ expression. It was observed that RGS4-LacZ was expressed primarily in the more contractile areas of the monolayer (Figure 9A). To examine whether RGS4 expression coincided with regions of the monolayer that contain mostly cardiomyocytes, RGS4-LacZ stained monolayers were immunostained stained for the cardiac myocyte marker cTNT. Indeed, RGS4-LacZ expression was found primarily in areas that were cTNT⁺ (Figure 9B-D). This suggested that other cell types that had not differentiated into cardiomyocytes, including but not limited to smooth muscle cells and hematopoetic cells which do not express high levels of the RGS4 mRNA. To further determine whether RGS4-LacZ expression may also overlap with areas that contain pacemaker-type cells, the cardiac monolayers were co-stained for LacZ and HCN4. As shown in Figure 9E, RGS4 expression was often localized to regions that were HCN4⁺.
**Figure 9.** RGS4 is expressed on cardiomyocytes differentiated in monolayers. (A) Bright field image of RGS4 expression (indicated by the blue-LacZ stain) on cardiomyocytes with a colour camera. (B) Bright field image of RGS4-LacZ stain (black dots indicate Rgs4-LacZ expression) on the fluorescent microscope. (C) Cardiac troponin stain to identify cardiomyocytes (Red) (C) Overlay of (B) and (C) illustrating RGS4-LacZ expression occurs specifically in cardiomyocytes. (E) HCN4 stain by IHC indicating the co-expression of RGS4 and HCN4. The first image is shown as a 10x, the enlarged image at 40x shows the co-stain of LacZ with HCN4 stain.
3.b.iv. Time-course showing onset of RGS4 expression

To determine the onset of RGS4 expression during mESC differentiation to cardiomyocytes, after sorting cell populations at Day 3+3h for Flk-1⁺/PDGFR-α⁺ and plating this population as a monolayer for cardiac lineage specification, the monolayers were fixed daily and stained for RGS4-LacZ with β-galactosidase. It was observed that RGS4-LacZ expression was upregulated at Day 5 with increasing levels of RGS4-LacZ expression as cardiomyocytes were differentiated. It should be noted that spontaneous contraction of the monolayers occurs between Day 6 and Day 7 of differentiation, about a day after RGS4 expression is first observed by LacZ staining.

FIGURE 10

Figure 10. Time-course showing onset of RGS4-LacZ expression. RGS4 mES cell monolayers. (A) Day 4—One day after plating (60’000 cells per well in a 96-well flat-bottom plate). No RGS4 expression was observed. (B) Day 5—cardiomyocytes start to form as observed by the “pillowing” effect of the cells. Some RGS4 expression is observed (yellow arrow). (C) Day 6—monolayers of cardiomyocytes begin contracting. Increase in RGS4 expression from previous day is observed. (D-E) Day 9 and 11—monolayer is steadily contracting with noticeable RGS4 expression. (F) RGS4 expression of a Day 11 monolayer illustrating RGS4 expression only in the cardiomyocytes.
3.c. Stem cells: RGS4-GFP line

Due to the limitations of the RGS4-LacZ cell line (described in the Discussion section), a RGS4-GFP cell line was derived by the Toronto Centre for Phenogenomics from RGS4-GFP mice purchased from Jackson laboratories.

3.c.i. Optimizing the differentiation protocol

3.c.i.1. Determination of Activin A and BMP4 concentrations

The cytokines, Activin A and BMP4, used at the primitive streak and mesoderm induction stage of differentiation, are crucial for differentiation of mESCs into cardiomyocytes as previously described. As with every new cell line, the optimal ratios of Activin A and BMP4 need to be determined empirically to give the highest percentage of the Flk-1⁺/PdgfR-α⁺ cell populations. At this initial step, there were major challenges to increase the size of the Flk-1⁺/PdgfR-α⁺ cell populations. Furthermore, this particular cell line showed differences in doubling time and cell viability during the differentiation process compared to the RGS4-LacZ line and several other commonly used ES lines. Thus, the troubleshooting measures described below were necessary to optimize the differentiation protocols for this new line. As seen in Figure 11A, the cell count for each population was extremely low, which was due to the high level of cell death and slow cell proliferation rates during the differentiation process. In addition, it was observed that there were very low numbers of cells present in the Flk-1⁺/PdgfR-α⁺ cell population when the standard differentiation protocol was used. This posed a serious problem because it is known that cells that are not Flk-1⁺/PdgfR-α⁺ do not differentiate into cardiomyocytes. Thus, the first hurdle was to increase the size of the Flk-1⁺/PdgfR-α⁺ cell population.
FIGURE 11

Figure 11. Activin A and BMP4 grid for the RGS4-GFP mESC line. Optimal ratios of Activin A and BMP4 used for the induction of mouse embryonic stem cells (mES) were determined empirically to give the highest percentage of cardiac troponin T cells (cTNT) by measuring for double positive cells expressing Flk-1 and PDGFR-α. (A) Initial grid was shown that this cell line contained a lot of issues including cell proliferation and cell survival issues as well as the inability to increase the size of the Flk-1+/PDGFR-α population. (B) A subsequent grid was set up with the addition of Wnt3a to the differentiation media at Day 2 to boost the induction of mesoderm differentiation. Subtle increases were observed in comparison to the initial grid, sufficient to select 8 ng/ml of Activin A and 0.5 ng/ml of BMP4 as the ideal concentration to use for the induction of this cell line.
3.c.i.2. Effects of Wnt3a

Many factors were taken into consideration to uncover the reasons for the lack of cell proliferation, the increase in cell death of these cells and the absence of the Flk-1⁺/PdgfR-α⁺ cell population during the early stages of differentiation. At the early stages of differentiation, Wnt signaling plays a role in the development of the mesoderm germ layer. Wnt3a is usually not supplemented for the differentiation of the mESC into mesoderm. The endogenous expression of Wnt3a is generally sufficient for the differentiation. Given that this cell line was incapable of generating a sufficient size of Flk-1⁺/PdgfR-α⁺ cell population, it was suspected that this cell line may not have produced adequate amounts of Wnt3a to support the Wnt signaling pathway required for the differentiation of these cells. Thus, during differentiation at Day 2 the media for the induction of the mesoderm was supplemented with varying concentrations of Wnt3a (0 – 50 ng/ml) (Figure 12). Although the media supplemented with Wnt3a did not show significant improvements with increasing the size of the Flk-1⁺/PdgfR-α⁺ cell population, nor did it show significant improvements with cell proliferation (Figure12A and B), there was a slight increase in the Flk-1⁺/PdgfR-α⁺ cell population size. Given that there is no harm and had some benefit with the addition of Wnt3a to the media, subsequent experiments were supplemented with 10 ng/ml Wnt3a at Day 2 to boost the size of the Flk-1⁺/PdgfR-α⁺ cell population.

3.c.i.3. Effects of ROCK Inhibitor Y-27632

To target the issue of cell survival, the effects of the Y-27632, a selective inhibitor of the p160-Rho associated coiled kinase (ROCK), were tested to determine whether it has beneficial effects to improve the recovery and survival of mESCs following differentiation induction. It was shown in a study by Emre et al.\textsuperscript{136} that the application of 10 μM ROCK inhibitor Y-27632
(RI) improved cell recovery with no effects on the pluripotency of human ES cells. To see if there are added benefits of RI on mESC differentiation or proliferation, RI was added to the differentiation media at Day 0 or Day 2 when the cells are dissociated and reaggregated in the differentiation media or at both time points. There seemed to be little observed benefits on improving cell survival and negligible benefit in increasing the size of the Flk-1⁺/PdgfR-α⁺ cell population when analyzed at Day 3 + 3h (Figure 12B). Thus, given the little added benefits of RI on cell survival and the unknown effects on the pluripotency on mESC, as it has only been tested on human ES cells, RI was not supplemented to the media in subsequent experiments.

Following the tests with Wnt3a and RI, it was decided that the standard media be supplemented with only Wnt3a. At this point, although the results were not optimal for a proper differentiation to the cardiovascular lineage, an Activin A and BMP4 grid was done, as seen in Figure 11B, to provide a general idea of the ideal concentration to use to continue with further testing. The addition of Wnt3a at Day 2 of the differentiation was sufficient enough to determine the optimal concentrations of factors that provided the highest percentage of Flk-1⁺/PdgfR-α⁺ population which was 8 ng/ml of Activin A and 0.5 ng/ml of BMP4. The determined concentrations were used for subsequent experiments.
FIGURE 12

Figure 12. The effects of Wnt3a and ROCK inhibitor Y-27632 (RI) on increasing the size of the Flk-1⁺/PdgfR-α⁺ cell population, cell survival and proliferation on the newly derived RGS4-GFP mESC from B6 mouse background. (A) Testing the effects of supplementing the media with varying concentrations of Wnt3a (0 - 50ng/ml) at Day 2 of differentiation to induce mesoderm cell population to increase the size of the Flk-1⁺/PdgfR-α⁺ cell population. (B) Adding combinations of Wnt3a and RI to improve cell survival and to increase the size of the Flk-1⁺/PdgfR-α⁺ cell population.
3.c.i.4. Delayed up-regulation of Flk-1 and PdgfR-α

The use of 2i inhibitors to culture the mESCs rather than the use of BMP4 was next hypothesized to have delayed the differentiation kinetics of the cell line. To test this theory, a kinetics experiment designed to capture the window of the upregulation of the Flk-1+/PdgfR-α+ cell population was performed. Starting at the standardized protocol timeline at Day 3 + 3h, samples of cells were analyzed for the size in the Flk-1+/PdgfR-α+ cell population. At every subsequent 3h time interval, samples of cells were subjected to FACS analysis for Flk-1+/PdgfR-α+ upregulation. With every tested time-frame an increase in the size of the Flk-1+/PdgfR-α+ cell population was observed (Figure 13A). Finally, with an extra half day to the standardized protocol at Day 3 + 15h, a significant increase in the size of the Flk-1+/PdgfR-α+ cell population was observed with the Flk-1+/PdgfR-α+ population, reaching more than 60% of the entire EBs in cultures. Although this finding solved the major issue of the inability to increase the size of the Flk-1+/PdgfR-α+ cell population, another issue still remained. With the minimal amounts of cells present at Day 3 the increased cell death at the induction of differentiation and the slow rate of cell proliferation both remained a standing issue.

3.c.i.5. Cell death and cell proliferation

To decrease the amount of cell death incurred during the differentiation process, the EBs formed at Day 0 to Day 2 were kept intact at Day 2. In the standard protocol, the EBs were generated and culture for 2 days in serum free media with no supplemented factors. These EBs would then be harvested and subjected to dissociation and reaggregation in serum-free media supplemented with VEGF, Activin A and BMP4. Since massive cell death was observed following the dissociation and reaggregation at Day 2, we compared cell survival between EBs
kept intact at Day 2 to cell survival from EBs that were dissociated and reaggregated. Using the newly revised timeline for the differentiation, the cells were analyzed at Day 3 + 15h by FACS. When the cells were subjected to dissociation and reaggregation at Day 2, the cell yield was at 4.5% with 21% Flk-1+/PDGFR-α+ (Figure 13B). When the cells were kept in intact EBs at Day 2, the cell yield was significantly higher at 40% and the Flk-1+/PDGFR-α+ was greater than 30% of the entire EBs in culture (Figure 13C). Thus, it seems that the dissociation of the cells at Day 2 caused increase in cell death due to the cells inability to recover from the dissociation. By keeping the cells intact, the percentage of live cells increased significantly such that there was sufficient amount of cells for continued differentiation into the cardiovascular lineage.

To address the concern of slower proliferation rates in this particular cell line during the induction of differentiation, the number of cells in the start-up culture was increased by five-times the previous start-up culture to ensure sufficient number cells were present for the differentiation.

With the optimized protocol (see optimized protocol in Figure 5) including all the modifications tested to improve the differentiation of this cell line, successful differentiations of this cell line was achieved. Sufficient cell counts and sufficient Flk-1+/PDGFR-α+ population was achieved (Figure 13D). Subsequent analysis to determine the efficiency of cardiovascular lineage differentiation by measuring cTNT by FACS also illustrated the success in differentiation with almost 45% of cTNT positive in the end culture of differentiation (Figure 13E).
Figure 13. Optimizing the differentiation of mESC protocol. (A) A kinetics study to capture the window of time for the upregulation of Flk-1⁺/Pdgfr-α⁺. (B-C) Effects of dissociating at Day 2 of differentiation. (B) EBs dissociated at Day 2 of differentiation following standard protocol achieving 21% Flk-1⁺/PDGFR⁺ with 4.5% live cells. (C) EB’s kept intact at Day 2 of differentiation achieving 62% Flk-1⁺/PDGFR⁺ with 40% live cells. (D-E) Success in the optimization of the protocol to achieve high levels of Flk-1⁺/PDGFR⁺ as well as the production of cardiomyocytes as measured by cTNT.
3.c.ii. Onset of RGS4 expression during differentiation

To determine the onset of RGS4 expression during differentiation, daily samples of RNA were extracted from the cells to measure RGS4 expression. It should be noted that the EBs in culture began to spontaneously contract at Day 7 of the differentiation process. Observed from qPCR analysis, RGS4 expression is upregulated at Day 6 of the differentiation process, one day prior to the observed spontaneous contraction of the EBs in culture (Figure 14). The upregulation of RGS4 coincides with the emergence of NKX2.5, a positive cardiac precursor, indicating that RGS4 expression is upregulated at around the same time cardiac precursors are formed. In addition, the emergence of RGS4 expression also coincides with HCN4, a channel highly expressed in pacemaker cells and plays a role in the regulation of the If current which is required for the pacemaking potential of the SAN.

Collectively, this shows that the expression of RGS4 is a unique component of the cardiac lineage in mESC cultures given its co-emergence of expression with NKX2.5 precursors and the pacemaking tissue marker HCN4.
Figure 14. Onset of RGS4 expression during differentiation in comparison to the expression of other markers. Quantitative PCR (qPCR) analysis of RGS4, HCN4, and NKX2.5 in RGS4-GFP derived embryoid bodies (EBs) at different stages during development. Bars represent mean ± standard error of the mean, n=3.
3.c.iii. Embryoid bodies expressing RGS4

To determine whether an association exists between RGS4 expression and the contraction of the embryoid bodies, a blinded microscopy-based experiment was conducted. In brightfield mode, EBs were selected and were recorded as contractile or not contractile. The setting was subsequently switched to the GFP filter and the EBs were recorded for expression of RGS4-GFP (Figure 16A). Forty-five spontaneously contracting EBs were recorded and 98% of those were RGS4-GFP expressing. Forty-five non-contracting EBs were recorded and 16% of those were RGS4-GFP expressing. This data indicates a strong correlation between RGS4 expression and spontaneous contraction.

3.c.iv. RGS4 expression in cardiomyocytes

RGS4 expression was analyzed by FACS based on GFP expression in differentiated cells. The cells were sorted between day 10-13 based on RGS4-GFP expression into a high-, med-, and low RGS4-GFP-expressing cell populations (Figure 15A). To determine if each of the populations post-sort varied in cardiomyocyte content, cTNT expression was measured by FACS analysis. The high-RGS4-GFP expressing cell population was 85% cTNT+ and was statistically significantly higher than the med- (57% cTNT+) and the non- (2% cTNT+) expressing cell populations. This provided us with an indication that RGS4 expression is highly correlated to cTNT+ cells such that those that do not express RGS4 in the non-RGS4-GFP expressing populations were very low in cardiomyocytes as indicated by the cTNT+.

This was further confirmed in culture where the morphology of the cells appeared to be very different from one another when the cells were re-plated in a 96-well plate post-sort based on the RGS4-GFP expression (Figure 15B-D). The high-RGS4-GFP expressing cell population
(Figure 15D), the cells formed relatively small clusters that spontaneously contracted at different rates. The med-RGS4-GFP expressing cell populations (Figure 15C) appeared to form relatively larger clusters that also spontaneously contracted at different rates. Cells in the non-RGS4-GFP expressing population do not form clusters (Figure 15B), but rather, they form a uniform monolayer of cells. The morphology and characteristics of the cells in the non-RGS4-GFP population looked significantly different than those seen in the RGS4-GFP-expressing cell populations such as the absence of cell clusters and the lack of contractility in the monolayers. As a result, the different morphology and observed characteristic of the monolayers in each of the cell populations suggest differences in the composition of cell types present in the different post-sort cultures.
**FIGURE 15**

Figure 15. RGS4 GFP expression. (A) RGS4-GFP expressing cells were sorted into three populations, high RGS4-GFP-, med RGS4-GFP- and Non RGS4-GFP expressing cell populations. Each of the samples were plated in to a 96-well plate at 200’000 cells per well (B,C,D) the non-RGS4-GFP-, Med-RGS4-GFP-, and high RGS4-GFP expressing sorted cell population, respectively, shown in bright-field and in GFP filter.

**FIGURE 16**

Figure 16. Characteristics of RGS4-GFP expressing cells. (A) The correlation and relation between RGS4-GFP expression with spontaneous contraction of cardiomyocyte EBs in culture. (n= 45 EBs for each) (B) Cardiac troponin expression measured by FACs from three populations post-sort, high-RGS4-GFP-, med-RGS4-GFP-, Low or non-RGS4-GFP-expressing cell populations. Cardiac troponin was measured by FACS. Asterisks indicate statistical significance as determined by t-test, *** (p≤0.0001), n = 4.
3.c.v. Characteristics of RGS4-expressing cells—a comparison of expression profiles with other genetic markers

To determine the characteristics of RGS4 expressing cells, the post-sorted cell populations high-, med-, and non-RGS4-GFP expressing cells were analyzed for various genetic markers by quantitative PCR (qPCR). RGS4 mRNA expression was first measured to illustrate that the RGS4-GFP BAC transgenic model was faithfully marking RGS4 expression. As seen in Figure 17, the transgene appears to mark RGS4 expression since the endogenous RGS4 mRNA levels correlated well with GFP levels of high-, med-, and low- or non- RGS4-GFP expressing cells.

To determine if high expression of RGS4 in cardiomyocytes corresponds to SAN-like cells, the pacemaker tissue marker, HCN4 was measured in the post-sorted populations. The expression profile of HCN4 was very similar to the expression profile of RGS4 in each of the post-sort populations. The high-GFP expression group was statistically significant to the med- and low-GFP expressing cell population and the med-RGS4-GFP cell population was statistically significant to the low-RGS4-GFP cell population (Figure 17). Thus, it illustrates that there is a correlation between RGS4 expression and HCN4 expression indicating that highly expressing RGS4 cells could be SAN-like. The expression of both RGS4 and HCN4 in these cells also correlate with the extent of contracting monolayers observed in culture post-sort, further indicating a role for RGS4 in SAN cells.

If the GFP-marked populations produced a pure population of SAN-like cells one may have expected low expression of Cx40, Cx43 and Scn5a (atrial-specific). Although Cx43 showed significantly decreased expression in the high-RGS4-GFP cell population in comparison to the low- or non-RGS4-GFP cell population, Cx40 and Scn5a had no significant differences in
expression between the high- and the med-RGS4-GFP cell populations consistent with the possibility that cardiomyocytes present in the high-RGS4-GFP cell population were a mixture of cell types, possibly SAN-like cells and atrial-like cells.

To further examine whether the types of cell in the high-RGS4-GFP cell population displayed SAN-like characteristics, the gap junctions Cx45 and Cx30.2 that are known to be expressed in the SAN in mice due to its low-conductance were measured by qPCR in the post-sort populations. It was observed that there were no statistical significant differences between the high- and med-RGS4-GFP cell populations, indicating that in the high-RGS4-GFP cell population contained mixture of cell types, SAN-like and atrial like (Figure 17).

To further characterize the cells expressing RGS4, the T-box transcription factors TBX2, TBX3 and TBX5 were measured in each of the post-sort cell populations. TBX3 is expressed in developing as well as mature conduction system cells including the SAN however the qPCR analysis shows that there was no statistical significance between any of the post-sort cell populations indicating that TBX3 is not a specific marker for the SAN and that each of the post-sort cell populations contain conduction system cells. TBX2 is selectively expressed in nonchamber myocardium, particularly in the atrioventricular canal. The qPCR analysis from the post-sorted cell populations showed some correlation with RGS4 expression such that the high-GFP is statistically extremely significant from the low-GFP cell population. TBX5 is involved in the regulation of TBX3 and SHOX2 in the development of the SAN and is expressed in the atrial and venousus myocardium. Given its role in regulating genes involved in the development of the SAN, it was of interest to note any correlation in its expression with RGS4. Although the high-GFP post-sort cell population was statistically significantly different from the low-GFP cell population, it does not seem to correlate with RGS4 expression.
Collectively, although the TBX2, TBX3 and TBX5 all play major roles in the development of the SAN, they do not seem to be specific enough to characterize whether or not high expressing RGS4 cells are SAN-like.
Figure 17. Quantitative PCR (qPCR) analysis of RGS4, HCN4, TBX3, TBX5, CX40, Cx30.2, Cx45, Cx43, αMHC (MYH6.1), βMHC (MYH7), HCN1, MLC2v (MYL2), SCN5a, and IRX4 in RGS4-GFP sorted cells based on RGS4-GFP expression levels. cTNT was analyzed by FACS analysis following the sort and is represented by percentage of expression in the population. Bars represent ± standard error of the mean. Asterisks indicate statistical significance as determined by t-test, * (p≤0.05), ** (p≤0.01), *** (p≤0.001), n=4.
3.c.vi. Patch clamp analysis on cardiomyocytes sorted by RGS4 expression

To determine the electrophysiological characteristics of cells that express RGS4, following the sort of differentiated mESC into high-, med-, and non-RGS4-GFP expressing cells, the cell populations were subjected to a blinded experiment and patch clamped. Cells were identified as producing spontaneous action potentials or not. In the cells that produced action potentials the shape of the action potential typically fell into one of two different classes. In the first class, the action potentials were similar to those of large cell or peripheral SAN cells (Figure 18A compared to Figure 1). In the second class, the action potentials appeared similar to those of the small cell or central SAN cells (Figure 18B compared to Figure 1).

When the ability of different sorted cell populations to produce action potentials was examined, it was noted that 80% (12/15) of the cells patched in the high-GFP cell population elicited spontaneous action potentials with 50% (6/12) of those contained the central SAN-like action potentials (Figure 18C). In the med-GFP cell population 50% (4/8) of the cells patched had spontaneous action potentials with 75% (3/4) of those with central SAN-like action potentials. In the non-RGS4-GFP expressing cells 14% (1/7) elicited a spontaneous action potential which was central SAN-like. Accordingly, it seems that the high RGS4-expression group correlates best with having spontaneous action potentials, most of them central-SAN-like. Hence, it can be said that RGS4 expressing cells seems to have the best potential to differentiate into cells with characteristics of central-SAN-like cells.
Figure 18. Electrophysiological characteristics of RGS4 expressing cells. Two types of spontaneous action potentials were recorded from the sorted cell populations: (a) two-phase-like action potential, (b) a more sinoatrial node-like action potential. (c) The percentage of cells with spontaneous action potentials. In the low RGS4-GFP expressing population (n=7 cells), 1 cell elicited spontaneous action potentials which had SAN-like action potentials. In the medium RGS4-GFP expressing populations (n=8 cells), 4 of the patched cells elicited spontaneous action potentials where 3 of them had SAN-like APs. In the High RGS4-GFP expressing cell populations (n=15 cells), 12 of the cells elicited spontaneous action potentials where 6 of them had SAN-like APs.
Chapter 4

Discussion

4.a. *In vivo* expression of RGS4 in the mouse model

RGS4-LacZ expression in the SAN was first observed at E11.5. As might be expected, there are some differences observed in the timing of onset of RGS4 expression relative to other SAN markers, in the *in vivo* mouse model and differentiated mESCs in culture. mRNA analysis of the mESC differentiation, showed the onset of RGS4 expression and HCN4 expression was coincident and occurred prior to spontaneous contractions. Consistent with previous reports that HCN4 expression is upregulated between E9.5-E10 *in vivo*30, our immunohistochemistry data showed that indeed HCN4 is prominently expressed by E10.5. The differences in observed expression may be potentially due to the fact that LacZ expression must exceed a certain threshold within SAN myocytes before its activity can be detected by our staining protocols. Thus, it is possible that RGS4 expression may have turned on at an earlier time points but we could not detect it (to be discussed in detail in the Limitations section). Future studies might employ laser capture microdissection to isolate mouse embryonic SAN tissue and measure RGS4 upregulation via qPCR.

To determine whether RGS4 plays a role in SAN development, the onset of RGS4 expression during development relative to the onset of contractile activity in cardiomyocytes was observed. In mESC cultures, RGS4 expression was upregulated just prior to spontaneous contractions of cardiomyocytes were observed. During murine cardiovascular development, the first sign of cardiac contractive activity was observed at E8.25, though those contractions were limited and weak, but by E10.5 the contractions were highly coordinated137. So it is clear that
the observed onset of RGS4 expression in vivo (at E11.5) arose well after contractile activity was established in in vivo cardiac development. As discussed above the apparent differences in the timeline of RGS4 expression relative to initiation of contraction may be due to limitations in our ability to detect the onset of RGS4 expression using the LacZ reporter. Another explanation for this difference is that it may be difficult to recapitulate the physiologic setting of sinoatrial node development using our in vitro mESC model and that the timing of RGS4 expression is highly dependent on physiologic cues from the developing heart. Thus, it remains to be determined whether RGS4 plays a role in the initiation of contraction based on the differing results obtained from the in vivo and in vitro model.

Another potentially important observation is that RGS4 expression in the SAN is localized to regions that are HCN4+ only. However, it should be noted that the observed area that is HCN4+ was often larger than tissue that is RGS4-LacZ positive (Figure 6). This observation is consistent with what was observed in the adult mouse as described by Cifelli et al. Thus, even throughout development RGS4 appears to be highly selective for sinoatrial node myocytes.

4.b. The use of RGS4-LacZ mESC to RGS4-GFP mESC

Initial mESC experiments were completed using the RGS4-LacZ cell line which proved to be extremely limited due to its lack of versatility for studying the characteristics of cells that were expressing RGS4. In order to visualize RGS4 expression in these cells, the cells required fixation followed by a β-galactosidase stain. Thus, it was impossible to isolate these cells as live cells to study the characteristics of RGS4 expressing cells. It was thought possible to sort these cells live through the use of a LacZ-flow cytometry kit (FluoReporter, Molecular
PROBES) which uses the fluorogenic β-galactosidase substrate fluorescein di β-D-galactopyranoside (FDG) in conjunction with FACS to be able to sort live cells based on β-galactosidase activity. This kit was extremely troublesome and was not very versatile. A few trials used to measure RGS4-LacZ expression by FACS have shown it to produce unreliable data through various experiments. The negative control which contained no β-galactosidase activity should be ideally 0% positive for RGS4-LacZ expression but the measured expression from the negative control sample was shown to be higher than that of all the samples, consequently, nullifying all the data (data not shown). Through repeated experiments, similar results were obtained so it was obvious that this method could not be employed for subsequent experimentation. Consequently, in order for us to study isolated cells expressing RGS4 from a cardiomyocytes differentiated in culture an RGS4-GFP cell line was derived from RGS4-GFP mice obtained from Jackson Laboratories. The use of cells expressing a fluorescent marker allowed us to readily analyze and sort these cells based on RGS4 expression.

4.c. Methodology to optimize protocols when working with mESCs

Because of inherent difference between different mESC lines, optimization is required when working with any new mESC line. During the differentiation process using the directed differentiation of the mESCs to cardiomyocytes we’ve observed 4 distinct cell populations at day 3.5 of the differentiation: Flk-1+/PdgfR+, Flk-1+/PdgfR-, Flk-1-/PdgfR+ and Flk-1-/PdgfR- (i.e. see Figure 7). Our goal is to optimize our conditions to obtain optimal yield of a Flk-1+/PdgfR-α+ cell population because cardiomyocyte potential is known to be associated with this cell population. The Flk-1-/PdgfR-α- cell population likely indicative of the emergence of an endoderm cell population. In addition, the Flk-1+/PdgfR-α- cell population has been shown
by Kattman et al.\textsuperscript{129} that cells from this cell population generated few cardiomyocytes but gives rise to a substantial population that is indicative of a hematopoetic population. Thus, it was very important to minimize the percentage of cells in the other subpopulations and optimize the percentage of cells in the Flk-1\textsuperscript{+}/PdgfR-\(\alpha\)\textsuperscript{+} cell population to achieve successful cardiac lineage differentiation. However, it should be noted that with optimization of the protocol, it is inevitable that the cultures will contain still other cell types at the end of the differentiation, but as a minority of the cells in the culture.

The optimization of Flk-1\textsuperscript{+}/PdgfR-\(\alpha\)\textsuperscript{+} cell population is usually determined by a standard grid with increasing concentrations of Activin A and BMP4. However, with the new cell line denoted as RGS4-GFP derived from B6.Cg-Tg(Rgs4-EGFP)4Lvt/J mice, some unusual complications were experienced. Specifically, we noted the inability to increase the Flk-1\textsuperscript{+}/PdgfR-\(\alpha\)\textsuperscript{+} cell population, the lack of cell proliferation during differentiation, and the occurrence of massive cell death. To overcome the absence of the Flk-1\textsuperscript{+}/PdgfR-\(\alpha\)\textsuperscript{+} cell population at day 3, we first identified the molecular pathway required for the induction of the mesoderm and attempted to boost the differentiation by activating the pathways involved. The particular pathway required for the induction of mESCs into mesoderm is the Wnt pathway.

Normal the endogenous production of Wnt3a is sufficient to activate this pathway and no supplementation of this reagent is required. In this line Wnt3a supplementation was provided and found to modestly increase the Flk-1\textsuperscript{+}/PdgfR-\(\alpha\)\textsuperscript{+} cell population (from 0.24\% to 1.66\%), and thus its use was continued throughout the remainder of the optimization studies. However, this modest increase did not provide a large enough number of Flk-1\textsuperscript{+}/PdgfR-\(\alpha\)\textsuperscript{+} cells for differentiation and further optimization was required.
As discussed in the introduction, the use of serum-free media supplemented with LIF and BMP4 allows the expansion and growth of ESCs in the absence of FCS and feeder cells. The original protocol for cardiac differentiation was generated with cells expanded with SF media supplemented with LIF and BMP4. Due to recent improvements in the media by replacing the BMP4 and LIF supplemented media to the use of two inhibitors (2i): MEK inhibitor PD0325901 and GSK3 inhibitor CHIR99021. The major advantage of using 2i media was that it allowed for better differentiation to enhance the percentage of Flk-1+/PdgfR-α+ cell population. We wondered whether the use of the 2i supplemented media affected the timing of the differentiation protocol. Shown in Figure 13A various windows of time of differentiation were tested and a significant increase in Flk-1+/PdgfR-α+ expression was observed with an added 12 hours to the standard protocol at Day 3. With the issue of Flk-1+/PdgfR-α+ expression resolved, the problem of increased cell death and slower rate of cell proliferation was pursued.

Since the cells were subjected to dissociation and reaggregation at Day 2, it was suspected that the dissociation of the EBs at Day 2 was also causing an unacceptable level of cell death. Although the dissociation of these EBs used a relatively gentle type of trypsin solution, TrypLE, it nonetheless dissociated the cells and affected the integrity of the intercellular connections. Due to the sensitivity of these cells, these ESCs may have had difficulty recovering following the disruption of their intercellular associations resulting in cell dissociation induced apoptosis. Since the use of RI (ROCK inhibitor), shown by Emre et al.,136 improved the overall recovery of human ESC after FACS sorting and improved the survival of hESC-derived cardiomyocytes following dissociation, it was hypothesized that the use of RI following the dissociation of the mESCs may improve cellular recovery and decrease apoptosis. However, no improvements with cell survival were observed following the addition of RI to the
differentiation media of the mESCs. Thus, it may be that inhibition of ROCK to improve survival rates may be associated with an apoptotic pathway specific to human ESCs and not to mouse ESCs. The eventual eradication of the dissociation step turned out to be the most effective way to decrease cell death in these experiments. Thus, from that point cells were not dissociated and reaggregated at Day 2 and the EBs remained intact until the final plating stage. As shown in Figure 13 (B-E) this modification significantly improved the survival rates of the cells in culture. Leaving the EBs intact at Day 2 of the differentiation contains some disadvantages. For one, the inability to maximize the surface area of the cell’s exposure to the differentiation media may affect the extent of differentiation of the cells at the core of the EB. In addition, the disadvantage of culture and differentiating ESCs in EB form is that the production of cytokines and inducing factors inside of the EB can complicate the interpretation of the experiment when understand lineage commitment. Thus, the samples would contain cells that may not have been differentiated into the cardiac lineage. However the benefits to the culture of leaving the EBs intact during differentiation significantly outweigh the disadvantages. Finally, because of the much slower rate of cellular proliferation for the GFP line, the start-up cell count for induction of mESCs was dramatically increased to reach the number of cells desired at the final replating stage.

By refining the process of differentiation for this particular cell line, it optimized the standard protocol to resolve the issues involved with the RGS4-GFP cell line and allow for proper mESC differentiation to the cardiac lineage.
4.d. RGS4 expressing cells are SAN-like

The foundation of this project was based on previous research illustrating the high and selective expression of RGS4 in the SAN and the role RGS4 plays in regulating the sinus rhythm due to its inhibition of parasympathetic signaling in mice. At the cellular level, we have illustrated that the correlation of cells expressing high levels of RGS4 with SAN-cell characteristics. Preliminary work with RGS4-LacZ mESCs illustrate and confirm that RGS4 is expressed in developing cardiomyocytes. Its expression was highly correlated with cTNT+ expressing cells as well as HCN4+ cells. In addition, with the ability to visualize the expression of RGS4 in live cells with the RGS4-GFP cell line, we were able to demonstrate the high correlation between the RGS4 expression and contractility of the cells. Majority of the cells that were contractile were RGS4 expressing and most of the non-contractile EBs were not RGS4 expressing. The presence of RGS4 expression in some of the non-contractile EBs may be due to the presence of other cell types or that there were minute contractions in the EB but at an unseen layer in the EB. Together, it suggests that the expression of RGS4 is associated with the appearance of contractile cardiac cells. It will be interesting to determine whether its expression was indeed high those contracting cells or instead whether it was highly expressed in a small number of SAN-like cells found associated with each contractile unit.

While it is known that there is a characteristic set of genetic markers associated with SAN cells, we were interested in determining whether highly RGS4 expressing cells fit the SAN characteristics. When the RGS4-GFP mESC cell line differentiated into cardiomyocytes, it was possible to subject the cells to fluorescence-activated cell (FACS) sorting based on RGS4-GFP expression and study the characteristics of the cells based on RGS4-GFP expression. As previously described, the SAN would ideally express HCN4, TBX3 and the low conductance
gap junction markers, Cx45, and Cx30.2; and have low expression of the markers found in the atrial working myocardium including Cx40, Cx43 and Scn5a. Through real time qPCR analysis, we showed that the cells expressing high levels of RGS4 contain a number of important characteristics of SAN-like cells.

By measuring the level of expression of IRX4, MLC2V, Myh6.1 and Myh7 which are chamber specific markers provided us verification that the cells that were sorted for RGS4 positive were cardiac. Given that we measured almost no RGS4 expression in the low-RGS4-GFP expressing cell population, the lack of expression of these markers in this cell population provided further confirmation that RGS4 expressing cells are associated with cardiac cells.

Sorted cells in the high-RGS4-GFP cell population, relative to the medium- and low-RGS4-GFP cell population have a similar pattern of expression when compared to HCN4 expression in these sorted populations. The expression profiles of RGS4 from the high-RGS4-GFP cell population and medium-RGS4-GFP cell population have a very similar expression profile observed from isolated SAN and right atrial appendage tissue, respectively, from isolated in vivo mouse tissue by Cifelli et al.\textsuperscript{116}. This also holds true for HCN4 expression profile, when the post-sort populations were compared to the work by Herrmann \textit{et al.}\textsuperscript{138}. They showed that the primary pacemaker center had significantly higher HCN4 expression than in the right atrium similar to what was measured in the high- and medium-RGS4-GFP cell population, respectively.

Another marker indicative that these cells are potentially SAN is the qPCR expression profile of Cx43 on post-sort cell populations. The high-RGS4-GFP cell population was statistically significantly lower from the low-GFP cell population which corresponds very well with the profile that SAN cells have low expression of CX43. This coincides with previous
finding in various mammalian SA nodes where Cx43 expression was not detected.

Furthermore, since our differentiated cell populations includes a variety of cells that arise from the mesoderm cell population, it wouldn’t be surprising to have hematopoietic cells in our cultures. Since the cell types in the low-RGS4-GFP expressing cell population have almost no cTNT positive cells which indicate that these cell types are not cardiac. Thus, we suspect this population of cells to contain other cell types including hematopoietic cells, which would explain the high expression of Cx43 found in the low-RGS4-GFP expressing fraction since it has been shown that Cx43 expression is critical for normal hematopoiesis\textsuperscript{139}. In addition, the expression pattern of Cx40, a fellow high-conductance gap junction lowly expressed in SAN cells in vivo seems to elicit a trend indicative of SAN-like cells in the high-RGS4-GFP expressing cell population. Although not statistically significant, the expression in the med-RGS4-GFP expressing cell population has higher expression of Cx40 when compared to the high-RGS4-GFP expressing cell population.

In addition, it was expected that there would be high expression of the low conductance gap junctions Cx45 and Cx30.2 in the SAN-like cells found in the high-RGS4-GFP expressing cell population. Although there was no statistical significance between the groups to indicate higher expression in the high group in comparison to the med-group, the trend of the expression pattern illustrates that the expression in the high-RGS4-GFP expressing cell population is higher than that in the med-RGS4-GFP expressing cell population. Together with the expression profile of HCN4 (Figure 17), it seems that these cells are potentially SAN but further studies are required to further characterize these cells.

A further look at the mRNA expression other SAN markers through qPCR analysis including TBX3 and Scn5a it appears that the expected expression pattern based on in vivo
cardiac expression was not observed for the high-, medium-, and low-RGS4 GFP expressing patterns. However, the reason for the lack of trends seen with these genetic markers can be explained by the method of sorting which is elucidated in the limitations section. Briefly, the arbitrary choice of gates selected to represent high-, medium-, and low-RGS4 GFP cell populations may play a role in the inability to establish distinct expression pattern of known cardiac genetic markers. It is unknown as to how much RGS4 expression is required for a true representation of a SAN cell population. Thus, this alone can be a factor in our inability to capture the differences in genetic marker expression between the different sorted cell populations. In addition, it is not surprising that we were not able generate the ideal and expected pattern of expression of some genetic markers due to the differences and the ability to idealistically mimic the in vivo conditions in culture.

It was of interest to note that during differentiation the upregulation of RGS4 corresponds to the upregulation of NKX2.5 and HCN4. Early expression of HCN4 and NKX2.5 play important roles in regulating the development of the heart. Given the areas in which RGS4 is expressed and that its upregulation of expression coincides with the upregulation of HCN4 and NKX2.5, it would seem that RGS4 may play a role in the organization of SAN and atrial development. The role of RGS4 during this developmental step has yet to be discovered. However, a useful application of the expression of RGS4 during the maturation of cardiomyocytes allows for the purification of cell populations enriched with node-like properties. As seen from the high HCN4 expression in the high-RGS4-GFP expressing cell population and the high percentage of cells that elicited spontaneous action potentials in the cell population, it seems promising to utilize high RGS4 expression as a marker for purification of SAN myocytes.
Another interesting observation is the increase in RGS4 expression at later time point, Day 18 (Figure 14). The early expression of RGS4 during differentiation may indicate a regulatory role in the development of the SAN and atrial working myocardium, whereas the maintenance and continued expression of RGS4 may indicate a role in regulating the contraction of the EBs. However, the increase in expression noted at the later time point may indicate a different role for RGS4 in the cardiomyocytes in culture as the EBs mature. Further analysis is required to study the long-term role of RGS4.

Also, the RGS4-expressing cells were characterized by comparing the electrophysiological characteristics of the cell types found in each of the post-sort cell populations by patch clamping. It was observed that there were two types of action potentials observed, one was similar to the action potentials of large cell or peripheral SAN cells and the other was similar to the action potential of the small cell or central SAN cells. Because 90% of the cells patched in the high-GFP cell population elicited spontaneous action potentials and only 50% of those patched in the med-GFP cell population, there seems to be a strong correlation between the level of RGS4 expression and the extent of spontaneous action potentials observed. In addition, there is high probability that high RGS4 expression is associated with inner or small SAN cells, since we see a high percentage of the small SAN cell type action potential in the high-RGS4 expressing cell population. It should be noted that, a previous colleague from our lab noted that in mice, high levels of RGS4 expression on a group of small cells of tightly packed connective tissue resembling spider cells, which was thought to have been central SAN-type cells. This indicates that RGS4 may potentially play a role in regulating the autorhythmicity of small SAN cells and to a lesser extent regulates large SAN cells (personal communication with Carlo Cifelli).
Collectively, the data seems to support the hypothesis that high RGS4 expressing cells are SAN-like cells. Additional characterization of high-RGS4-expressing cells needs to be carried out for confirmation of the cell types that are SAN cells in mESC differentiated cardiomyocytes.

**4.e. Limitations**

A limitation that should be taken into consideration is that LacZ expression must exceed a specific threshold before its activity can be detected by staining protocols, which can confound observed expression location. This threshold was exemplified from qPCR data. For example, the mRNA data from Cifelli *et al.*\textsuperscript{116} illustrated that RGS4 is expressed throughout the heart but with varied levels of expression such that the SAN, which is stained for LacZ\textsuperscript{+}, expresses RGS4 4-fold higher than that of the atria. The atria is not stained as LacZ\textsuperscript{+}, but has been determined by qPCR to express low levels of RGS4. Consequently, the use of LacZ as a reporter for my studies as an indicator of RGS4 upregulation during development is not ideal since it is highly possible to miss the onset of expression due to low expression.

Another limitation is that during the differentiation of the RGS4-GFP line, in order to minimize the severe amounts of cell death, the EBs were not subjected to dissociation and reaggregation at Day 2 of the differentiation. The main disadvantage is inability to maximize the surface area of the cells’ exposure to the differentiation media thus affecting the extent of differentiation of the cells at the core of the EB. In addition, as previously mentioned, the disadvantage of culturing and differentiation ESCs in EBs is that the production of cytokines and inducing factors inside of the EBs can complicate the interpretation of the experiment when understanding lineage commitment. Thus, being unable to control the types of cytokines and
inducing factors released inside the EBs, it is hard to control lineage differentiation of those cells. That being said, the first time that these cells were dissociated for analysis was for the FACS sort based on RGS4-GFP expression. So, the samples would then contain cells that may not have been differentiated into the cardiac lineage. Those cells that were cTNT can contain a large variety of unknown cell types with unknown properties, potentially confounding the explanation of the data found in each of the post-sort populations.

An additional limitation is that the timelines for mESC differentiation cannot be linked back to the timelines of *in vivo* development. The differentiated cells are never considered as mature cells, the level of maturation of differentiated cells is unknown and cannot be correlate to *in vivo* development. Thus, if changes in expression occur from early development to maturation, it is impossible to link the observations from mESC differentiation to in utero development. Although it has been observed that RGS4 expression is highly and selectively expressed in the SAN in the mouse by E14.5 and is continually expressed in this manner throughout adulthood, it is hard to know at what stage of mESC differentiation corresponds to E14.5 in the mouse. Hence, some reservations are required for any conclusions drawn from the mESC experiments.

Finally, another limitation of the mESC experiments lies in the sorting of RGS4-GFP cardiomyocytes. The gating for each of the cell population was limited such that each population was required to contain a minimum percentage of cells to ensure sufficient cell retrieval. It would be ideal to isolate for the extremely high RGS4-GFP expressing cells since it is those cells that are hypothesized to be SAN-like, however, the gating is limited to a minimum percentages of cells in each cell population. Thus, the sorted populations as high-RGS4-GFP cell population contain not only the highest expressing RGS4-GFP cells, but also contain those
cells that have slightly lower RGS4 expression which should technically belong to the med-RGS4-GFP cell population. Due to the technical limitations, it was not possible to study the ideal cells in our hypothesis which may result in confounding data.

**4.f. Future directions**

Given that the expression of RGS4 upregulation in the mESC differentiation was found to coincide with the upregulation of HCN4 and NKX2.5, it would be of interest to determine the molecular role of RGS4 during SAN and atrial development. NKX2.5 plays a crucial role in repressing TBX3 and HCN4 which results in the upregulation of atrial working myocardium genes including Cx40, Cx43 and Scn5a. The specific location of NKX2.5 expression during atrial and SAN development defines fate of the cells as SAN or atrial working myocardium. With RGS4 expression upregulated together with NKX2.5 and HCN4, it can be assumed that RGS4 has a role, whether it is regulated by these genes or regulates these genes, during the development of the SAN and atrial working myocardium. To elucidate this role would aid and enhance our general understanding of cardiovascular development.

In addition, it was observed during the kinetics study that RGS4 expression is upregulated as the mESCs mature (Figure 14). It would be of interest to determine whether cells expressing RGS4 later during development are characteristically different than those expressed earlier as studied in this thesis. An analysis of atrial and SAN genetic markers on post-sorted cells based on level of RGS4 expression can provide details as to whether the characteristics of the cell types change during development. This would provide us an indication whether the role RGS4 plays early in development is similar to the role it plays in mature SAN.
In the future it would be beneficial to generate an RGS4-GFP human ESC cell line, to study the RGS4 in human development to bring the research closer to clinical practices. This would provide many answers and to adjoin the research on RGS4 discovered in mice and correlate it with human data. In addition, it would be highly useful to generate an mESC or hESC cell line that utilizes the tetracycline-controlled transcriptional activation for both a tet-on and tet-off element which allows for further characterization of differentiated cardiomyocytes by observing trends following the activation of RGS4 at various stages of cardiac lineage differentiation and in contrast observe the trends associated with turning off RGS4 expression at various stages of development. This would provide us with additional details on the roles RGS4 may play during development.
Chapter 5

References


Ref Type: Generic


137. Nishii K, Shibata Y. Mode and determination of the initial contraction stage in the mouse embryo heart. *Anat Embryol (Berl).* 2006;211:95-100.
