The Characterization of a Human Disease-Associated Mutation *Nkx2.5 R142C* Using *In vitro* and *In vivo* models

By

Abeer Zakariyah

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Department of Biochemistry, Microbiology and Immunology

Human Molecular Genetics

Faculty of Medicine

University of Ottawa

Abstract

Nkx2.5 is a cardiac transcription factor that plays a critical role in heart development. In humans, heterozygous mutations in the *NKX2.5* gene result in congenital heart defects (CHDs), but the molecular mechanisms by which these mutations cause the defects are still unknown. *NKX2.5 R142C* is a mutation that is found to be associated with atrial septal defect and atrioventricular block in 13 patients from one family. The *R142C* mutation is located within both the DNA-binding domain and the nuclear localization sequence of NKX2.5 protein. The pathogenesis of CHDs in humans with *R142C* point mutation is not well understood. Also, a previous study in our laboratory has identified Mypt1/PP1 as a novel interacting partner of Nkx2.5 in stem cells during cardiomyogenesis. Nkx2.5 has a PP1-binding consensus sequence RVxF located in the N-terminus of the homeodomain. Notably, the PP1-binding sequence, RVxF, is mutated from arginine to cysteine in patients with the *R142C* heterozygous mutation. However, the ability of the *R142C* mutation to bind to the Mypt1/PP1 complex has not been investigated yet. The following thesis addresses the functional deficit associated with *R142C* by utilizing a combination of *in vitro*, and *in vivo* models. It also addresses the interaction of Mypt1/PP1 with the *R142C* mutation. We have generated a heterozygous mouse embryonic stem cell (mESC) line, harboring the murine homologue (R141C) of the human mutation *R142C* in Nkx2.5 gene. We show reduced cardiomyogenesis and impaired subcellular localization of Nkx2.5 protein in $Nkx2.5^{R141C/+}$ mESCs. Gene expression profiling of

Nkx2.5$^{R141C/+}$ mESCs revealed a global misregulation of genes important for heart development and identified putative direct target genes of Nkx2.5 that are affected by the R141C heterozygous mutation. We also generated a mouse model harboring the human mutation *R142C*. We show that the *Nkx2.5*$^{R141C/R141C}$ homozygous embryos are developmentally arrested around E10.5 with delayed heart morphogenesis. Moreover, *Nkx2.5*$^{R141C/+}$ newborn mice are grossly normal but show variable cardiac defects and downregulation of ion channel genes that later cause AV block in adult mice. Finally, we show that the R141C mutant binds to the Mypt1/PP1 complex but is not inhibited or translocated to the perinuclear region in the presence of Mypt1/PP1 as the WT Nkx2.5 is.

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List of Abbreviation

Actc1 – Alpha cardiac actin

ANF – Atrial natriuretic factor

ASD – Atrial septal defect

AVSD – Atrial ventricular septal defect

AV – Atrioventricular

AVC – Atrioventricular canal

bHLH – Basic Helix-Loop-Helix

BMP – Bone Morphogenetic Protein

cDNA – Complementary deoxyribonucleic acid

CHDs – congenital heart defects

Cx40 – Connexin40

D – Day

DNA – Deoxyribonucleic acid

DORV – Double outlet right ventricle

EMT – Epithelial to mescenchymal transition

E – Embryonic day

EB – Embryoid bodies

EC – Endocardial cushion

ESC – Embryonic stem cells

Echo – Echogardiography

ECM – Extracellular matrix

Estrp71 – Ets-related protein 71

FBS – Fetal bovine serum

FGF – Fibroblast Growth Factor

FHF – First heart field

FOG – Friend of GATA

GATA – GATA-binding protein 4

GFP – green fluorescent protein

Hand1 – Heart and neural crest derivatives expressed transcript 1

HD – Homeodomain

IP – Immunoprecipitation

IRX – Iroquois homeobox

IVS – Interventricular septum

Kb – Kilobase

kDa – Kilodaltons

KO – Knockout

MEF2 – myocyte enhancer factor 2

Mesp – Mesoderm posterior homolog

mRNA – Messenger ribonucleic acid

MP – Myosin phosphatase

Mypt1 – Myosin phosphatase target subunit 1

MHC – Myosin heavy chain

mESCs – Mouse Embryonic Stem Cells

Nkx – NK transcription factor related

NK2-SD – NK2 specific domain

NLS – Nuclear localisation signal

OFT – Outflow tract

PBS – phosphate buffered saline

PP1 – Protein Phosphatase 1

PVDF – Polyvinylidene fluoride

QPCR – Quantitative Polymerase Chain Reaction

ROCK – Rho Associated Coiled-coil forming serine/threonine kinase

SA – Sinoatrial

SHF – Second heart field

Tbx – T-box transcription factor

TF – Transcription factor

TN – Transactivation domain

VSD – Ventricular Septal Defect

WT – Wild type

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CHAPTER 1

INTRODUCTION

This chapter provides a brief overview of the stages of heart development as well as examples of important signaling pathways and transcription factors involved in heart development. Moreover, it provides a brief overview of the congenital heart defects (CHDs) and the role of Nkx2.5 transcription factor in the development of these defects. It also gives an overview of the *in vitro* cardiomyogenesis in stem cells and signaling pathways and transcription factors involved in this process.

1.1 Heart Development

The heart is the first organ to form during embryogenesis and is responsible for pumping and supplying blood to the surrounding tissues to facilitate embryo growth (1). Heart development is a complex process that requires tightly regulated signaling pathways and cardiac transcription factors that turn on genes important for proper development of this organ (2). As the development of the embryo progresses, the heart is transformed from a primitive heart tube to a four-chambered heart (3). The stages of heart development include the formation of a linear heart tube, looping, chamber morphogenesis; septation and finally the formation of a mature four-chambered heart. These stages are discussed briefly in the following section (Figure 1.1).

1.1.1 Stages of heart development

The cells that will contribute to the vertebrate heart originate from the mesodermal progenitors, which leave the primitive streak and migrate laterally to form a crescent-like structure, called the “cardiac crescent”, below the head folds of the embryo (4, 5). The cardiac crescent is the first morphological sign of heart development and contains two distinct populations of cardiac progenitors: the first and the second heart fields (FHF and SHF). As development progresses, the FHF and SHF cells fuse at the embryonic midline to form the beating linear heart tube, which represent the first functional organ in the embryo (E8.0 in mice; day 21 of gestation in humans). The linear heart tube is composed of two layers: the endocardium that forms the inner endothelial layer of the heart; and the myocardium, forming the muscular wall of the heart. Both the endocardium and myocardium are separated by a space filled with extracellular matrix called cardiac jelly (6, 7). The FHF eventually contributes to the left ventricle and both the right and left atria (8). The SHF is the second source of myocardial cells is positioned anteriorly and dorsally to the tube and gives rise to the right ventricle and outflow tract of the heart (9–12).

The next stage of heart development is the rightward looping of the heart tube to ensure the correct position of the atria, ventricle chambers, aorta and pulmonary artery between days (E8.5-E10.5 in mouse). The linear heart tube undergoes a complex progression in which the tubular heart adopts a spiral shape and the atrial region is forced dorsally and located above the developing ventricles. Subsequently, a complex remodeling events, including a lengthening

of the tube and further ballooning that involves the expansion and bulging of the linear walls of the looped heart tube, takes place to form the four chambered heart. Upon looping, the linear heart tube creates an outer curvature and inner curvature, where the cavities of the definitive chambers are formed by the growth of the outer curvature (13).

As cardiac development progresses around day E9.5 in mice the right ventricle is connected to the primitive left ventricle by an atrioventricular canal (AVC). The endocardium cells located in the AVC and outflow tract (OFT) generate mesenchymal cells that migrate toward the cardiac jelly in a process called epithelial to mesenchymal transformation (EMT). The EMT is an important process for forming the endocardial cushion (EC) region that will subsequently form the septum, which is needed to form the four chambered heart, as well as both the tricuspid and mitral valves in the outflow region (14).

Another cardiac progenitor called the neural cardiac crest cells originate from hindbrain, migrate toward the outflow tract cushion and participate in cardiac septation (15, 16). At the same time, the myocardium in the ventricle wall starts to form trabeculation, which is a spongiform of myocytes in the inner layer of the ventricles that extend into the lumen. Trabeculation results in the increasing of myocardial mass and wall stiffness, which are important during chamber morphogenesis and for the growth of the heart. Trabeculation is also important to increase myocardial oxygenation and generate much of the contractile force of the heart that is necessary for ventricles contraction and distribution of electrical impulses (15, 17).

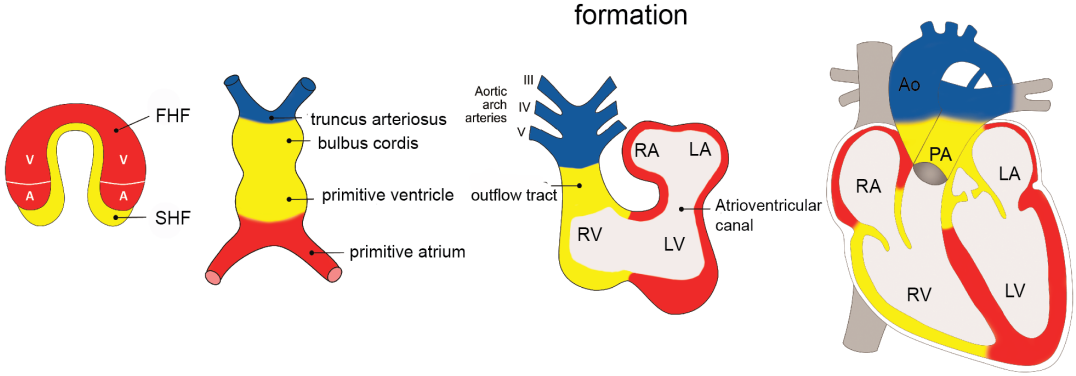
The outer layer of the heart covering the myocardium is called the epicardium, which is initially developed from a proepicardial organ. The proepicardial cells also invade the myocardium where they develop into coronary smooth muscle and fibroblast cells (18, 19). At the end of the looping stage, the heart contains a common atrial chamber, an atrioventricular canal (AVC), left and right ventricles and outflow tract (6, 15).

Conduction myocardium arises from the working myocardium. In early heart development, at the linear heart tube stage, there is no distinct region of a conduction system. However, the contractile impulse begins in the primary pacemaker region (the future sinus atrial (SA) node) located in the primitive atrium. The primary pacemaker region contains fast beating myocytes that send conduction impulses that spread toward the primitive ventricle and outflow tract (20). With further heart development, specialized cells of the cardiac conduction system proliferate and develop contractile properties. The first morphologically visible differentiation of the conduction system is the SA node, which is located in the wall of the right sinus horn in the right atrium. It functions as the pacemaker of the heart, transmitting the impulse through the atrial myocardium to the atrioventricular (AV) node. The AV node is located at the base of the atrial septum and is responsible for delaying the SA node impulse from the atrium to the ventricle myocardium to organize atrial diastole and ventricular systole (21, 22). The electrical impulse is then transmitted to the ventricular conduction network containing both conducting AV bundles (His bundle) and bundle branches and connected to Purkinje fibers that are formed from the trabecular

myocardium of the ventricle (22, 20). The SA node and AV node are developed from the slow conducting myocardium in the inflow tract and AV canal, whereas the AV bundle and bundle branches develop from the fast conducting ventricular myocardium (23). The Purkinje fibers are connected to cardiomyocytes through gap junction proteins such as connexins (24).

The development of the cardiac chambers involves septation and valve formation to give rise to a mature heart. Cardiac septation takes place after heart looping to ensure separation of the left and right sides of the atria and ventricles (25). Septation of the atria occurs when a ridge develops from its roof called the septum primum, which grows from the atrial wall toward the EC in the AVC. The opening between the septum primum and cushion is known as ostium primum, which is closed upon further growth. Before complete closure of the ostium primum, a small hole appears in the septum called ostium secundum. The ostium secundum allows the flow of blood from the right to left atrium, which is important for fetal circulation. While the septum primum grows, another septum grows to the right of the septum primum called septum secundum. The septum secundum does not completely close, leaving an opening called the foramen ovale, which closes soon after birth to create a complete septum. The remaining septum primum becomes the valve of the foramen ovale. Lastly, the septum secundum and septum primum fuse to form the atrial septum. Ventricular septation takes place when the tissue from the bottom of the ventricles called the interventricular septum (IVS) grows toward the endocardial cushion and separates the right and left ventricles (25–27).

The development of AV (mitral and tricuspid) and outflow tract (OFT) (aortic and pulmonary semilunar) valves are important for proper flow of blood between systemic and pulmonary circulatory systems as well as to prevent mixing oxygenated with deoxygenated blood. The first step of valvulogenesis is the formation of EC in the AV canal and OFT of the primitive looped heart, which further undergo extensive remodeling of the extracellular matrix (ECM) into layers rich with elastin and collagen (28). At the late stage of gestation, the valves start to function and become mature after birth (29). At birth, there is a switch in the cardiovascular circulation and the lungs become functional, which reduces the pulmonary pressure and promotes the closure of the ductus arteriosus, thus separating the pulmonary and systemic circulations. These changes will increase pressure in the left atrium and decrease it in the right atrium, which will stimulate the foramen ovale to completely close (18).

	Cardiac crescent	Linear heart tube	Looping and chamber formation	Mature heart
				
Mouse development	E7.5	E8.0	E9.0-10.5	E15.0-18
Human development	Day 15	Day 21	Day 28-32	Day 50-birth
Key events	-Myocyte differentiation -Migration to the midline	-First heart beat -Anterior-posterior and dorsal-ventral patterern-ing	-Looping to the right -Early chamber formation -Cushion formation -Trabeculation -Early conduction system formation	-Cushion remodeling -Conduction system
Transcription factors	GATA4 MESP1/2 NKX2.5	GATA4 GATA5	GATA4 NKX2.5 TBX5 MEF2C eHAND dHAND PITX2	GATA4 NKX2.5 TBX5 FOG-2 HEY2 TBX1 PITX2 CITED SOX4

This figure is modified from references (Martin et al. 2015; McCulley et al. 2012)

Figure 1.1 Morphogenesis of the vertebrate heart.

The diagram of heart development is shown with color-coding of contributing cell populations. The cardiac crescent stage forms the linear heart tube, which then undergoes looping followed by ballooning of regions that will form the chambers. Subsequently, a series of septation events results in a four-chambered heart with parallel systemic and pulmonary circulations. At the cardiac crescent stage, the two heart fields represent different cardiac precursors. The first heart field (FHF, red) contributes to the left ventricle, and both atriums. The second heart field (SHF, yellow) contributes to the right ventricle and outflow tract. Cardiac neural crest cells populate the aortic arch arteries (III, IV, and VI) and aortic sac (AS) that together contribute to specific segments of the mature aortic arch. The days of development for mice and human are shown with examples of critical transcription factors involved at each stage. Also, key events for each stage are shown. This figure is modified from references (30, 31).

1.1.2 Signaling pathways during heart development

Heart development begins with the induction of meseodermal cardiac precursor cells to become cardiogenic from the endoderm and neighboring tissues that secrete positive and negative-acting signaling pathways. Some of these positive-acting signaling pathways are BMP2, BMP4 (bone morphogenetic protein), FGF8 (fibroblast growth factor 8), Wnt11 and 5 (32–35). Among the negative-acting signaling pathways that control the induction of specific mesodermal cells are Wnt ligands such as Wnts 1, 3a and 8, and anti-BMPs such as Noggin (2, 36–38).

Wnt signals are expressed ubiquitously and can have different effects during all phases of heart development. Wnt ligands, frizzled receptors, or extracellular Wnt inhibitors are expressed in different tissues during development depending on the time of action (39). For example, during specification of cardiac precursor cells in the FHF, downregulation of Wnt/β-catenin signaling is essential (39, 40). In contrast, Wnt/β-catenin is important for Isl-1-positive SHF progenitor cells that contribute to outflow tract cushion, interventricular septum and right ventricular development (41). Loss of Wnt/ β-catenin signaling in the SHF in mice causes embryonic lethality as well as defects in the OFT and right ventricular with a decrease in Isl-1-positive progenitors and downregulation of Fgf10, Shh and the non-canonical Wnt ligand Wnt11, suggesting that canonical Wnt signaling acts upstream of other signaling pathways (42, 43). Moreover, canonical Wnt signaling mediated by Wnt3a is important for Mesp1 expression

and mesodermal induction in mice and mouse embryonic stem cells (44, 45). However, canonical Wnt signaling is inhibitory after mesodermal induction. The specification of cardiac progenitor cells from Mesp1 positive cells is potentiated by the Wnt inhibitor (Dkk1), which is a direct target of Mesp1(46).

BMP signaling is required for the early induction of mesodermal cells into cardiogenic precursors, chamber formation and septation of the heart (47). During early cardiomyogenesis, BMP signaling is essential for FHF progenitor specification. Ablation of the BMP ligand receptor (Bmpr1a) using $Mesp1^{cre}$, results in the absence of the cardiac crescent and the primitive ventricle (48). In addition to the role of BMP signaling in cardiac induction, BMPs have a major role at later stages of heart development during chamber formation and septation. BMP2 and BMP4 are expressed in the AV and OFT myocardia, respectively, and both are essential for EMT in the OFT and the AVC, which is important for septation and valve formation (49–52). BMP2 is important for the formation of the EC during heart development and cardiac-specific deletion of BMP2 in mice results in reduction of EMT and formation of the AV cushion (49). BMP10 is essential for regulating cardiac growth, ventricular trabeculation and chamber maturation. BMP10 knockout mice demonstrate absence of ventricle formation and die at E9.5-E10.5 (53). BMP10 is also required for maintaining normal expression levels of several key cardiac transcription factors (53, 54).

FGF8 is known to have an important role during early specification of cardiac mesoderm and is the major FGF ligand driving SHF development. Ablation of FGF8 in mice embryos results in multiple cardiac malformations that

are related to SHD defects (55, 56). Thus, FGF8 is required for SHF proliferation, specification and survival (57). Also, conditional ablation of FGF8 using different cre lines in mice has shown that it is required during gastrulation, cardiac looping and migration of neural crest (55, 58, 59).

1.1.3 Transcriptional Control of Heart Development

The signaling pathways responsible for cardiac induction stimulate transcription factors that are evolutionally conserved and critical for controlling gene expression during heart development. Development of the heart tube into the mature four-chambered heart requires multiple stages that depend on a unique and sophisticated genetic program (2). Several key cardiac transcription factors such as NKX2.5, GATA4, TBX5 and MEF2C respond to signaling pathways that guide heart morphogenesis. These transcription factors bind and cross regulate each other in order to activate genes or positively regulate each other's activity to control cardiac cell fates, differentiation, and contractile genes (2, 60, 61). Moreover, transcriptional repression of cardiac genes and negative feedback loops are important during heart development (61). Genome wide profiling of FHF and SHF murine progenitors from E9.5, has shown that multiple transcription factors are expressed in both heart fields and show distinct molecular signatures of these progenitor populations (62, 63). Moreover, microarray gene expression profiling has been used to provide an atlas of cardiogenic gene expression in different stages during heart development and adult stage (64, 65). Examples of key cardiac transcription factors and their role during heart development are discussed below.

1.1.3.1 Nkx2.5 transcription factor

Protein structure

Nkx2.5 is a member of the class I NK-2 family of transcription factors containing homeodomain (HD), which is evolutionary conserved from flies to humans (66, 67). Nkx2.5 contains four conserved domains: a transactivation domain (TN) near the amino terminus, a homeodomain (HD), an NK-2 specific domain (NK-2 SD) near the carboxyl terminus, and lastly, the sequence of the last amino acids in the carboxyl-terminus (GIRAW) which is highly conserved within the NK-2 family (68, 69) (Figure 1.2). The HD domain of Nkx2.5 protein contains 60-amino acid that binds to a consensus DNA motif, (TTAAGTG) through a helix-turn-helix motif of three alpha helices (I, II, III), and the helix alpha III ensures DNA binding specificity (70). The HD is important to mediate interactions with other transcription factors including GATA4, Tbx5 and Mef2c (71–74). The HD also contains the nuclear localization sequence (NLS) at the N terminus of the homeodomain (RRRRKPR) that is important for nuclear translocation (75). A unique characteristic of the NK-2 family is the tyrosine at position 54 (Tyr54) in the HD that mediates sequence-specific interaction with DNA residues (TAAG). A recent study identified another NLS in the C-terminus QNRRYKCKRQR, which is important for Nkx2.5 nuclear translocation (221). The NK-2 SD shows synergistic and cooperative binding to DNA as a homodimeric and heterodimeric complex, which is mediated through the C-terminal domain (70, 76). The NK-2 SD is a highly conserved domain and is thought to mediate

interaction with other proteins, and has inhibitory function where it masks the transcriptional activity in an *in vitro* reporter assays. The precise function of the TN domain is not clear yet (77–79).

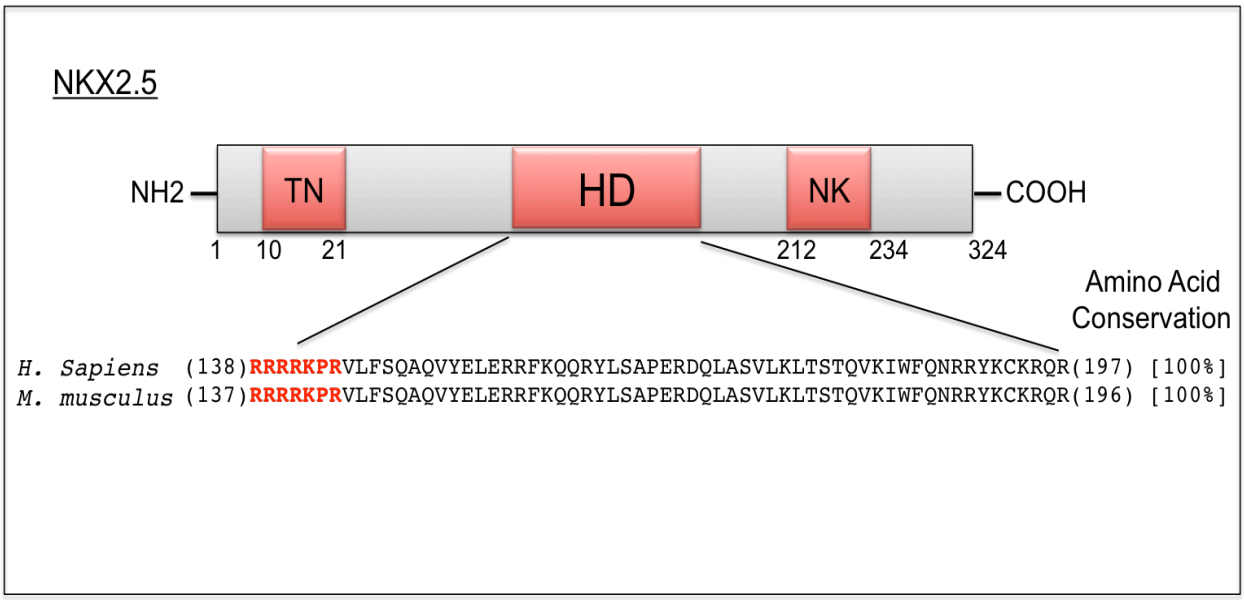


Figure 1.2 Schematic diagram of Nkx2.5 protein.

Functional domains of Nkx2.5 protein are shown: transactivation domain (TN) near the amino terminus (NH2), a homeodomain (HD), and NK-2 specific domain (NK) near The carboxyl-terminus (COOH). The sequence of the nuclear localization signal located in the N terminus of the HD is shown in red color.

Nkx2.5 regulation and interaction with transcription factors

Nkx2.5 regulates many of its target genes via direct interactions with other cardiac transcription factors such as GATA4, Tbx5 and Mef2c (71, 72, 80). Protein-protein interactions are important for the regulation of Nkx2.5 activity and thus, of cardiomyocyte differentiation and heart development. The interaction of Nkx2.5 with different cardiac transcription factors modulates its activity and confers target gene specificity during heart development. For example protein complex containing Nkx2.5 and other cardiac transcription factors could activate transcription, as seen for the atrial natriuretic factor (ANF) gene and alpha cardiac actin (ACTC1), or could inhibit transcription, as seen for β-catenin (74, 81–83). Also, cooperation of Nkx2.5 with Tbx5 or Tbx2 activate or inhibit expression of ANF, respectively (72, 84, 85). Also Nkx2.5 interacts with GATA4 and Tbx20 and synergistically stimulates ANF promoter (86).These findings highlight the importance of cofactor interactions for the regulation of Nkx2.5 activity.

The expression of Nkx2.5 is regulated by several transcription factors such as GATA4 and Mesp1 (87). Also, Nkx2.5 autoregulation is important for maintaining its own expression during SHF differentiation. In mice, the autoregulation of Nkx2.5 functions indirectly through Mef2c (88), whereas in chickens, Nkx2.5 binds directly to a genomic enhancer element that is required to maintain its expression in the SHF. Moreover, BMP is important for regulating Nkx2.5 expression because Nkx2.5 enhancer is composed of clustered repeats

of SMAD and GATA binding site, which is a direct targets of BMP (89). Nkx2.5 has multiple cis-regulatory elements; while one of the elements drives expression in the entire heart, two elements are specific for the right ventricle and outflow tract (90).

Nkx2.5 undergoes posttranscriptional modification such as phosphorylation and SUMOylation. The nuclear localization of Nkx2.5 is important for its phosphorylation at serine 163 (S163) by casein kinase II (CKII), which results in increased DNA binding and transcriptional activity of Nkx2.5 (75). Moreover, Nkx2.5 is targeted by SUMOylation at lysine 51 (K51) and the decrease in this process results in congenital heart defects in mice (91–93). A recent study has shown that Nkx2.5 is also involved in alternative polyadenylation and the regulation of the length of the 3' UTR where it interacts with 5'-3' exonuclease Xrn2 during heart development (94).

Nkx2.5 during heart development

Nkx2.5, also known as *Csx*, is one of the earliest transcription factors that is specifically expressed in the cardiac crescent at E7.5 and is maintained throughout adulthood (95–99). The importance of Nkx2.5 during heart development was first investigated in *Drosophila*. The *Tinman* gene is the Nkx2.5 homolog and is expressed in the developing dorsal vessel, which is equivalent to the heart in vertebrate. The absence of *Tinman* gene in *Drosophila* results in a loss of dorsal mesodermal derivatives, including the dorsal vessel, visceral muscle, and a subset of body wall muscles (100, 101).

The role of Nkx2.5 during heart development has been extensively investigated. In the murine model, germline deletion of Nkx2.5, results in embryonic lethality around E9.5-E10.0. The heart of the mutant embryo is arrested at the beginning of cardiac looping and there is an absence of ventricle trabeculation and EC formation (102–104). Also, in the null mutant embryo, cardiomyocytes are specified normally, but genes downstream of Nkx2.5, including the atrial natriuretic factor (ANF), the ventricular isoform of myosin light chain 2 (Mlc2v), Connexin40 (Cx40), eHAND/HAND1, N-myc and Iroquois homeobox gene 4 (Irx4) are aberrantly expressed (102, 103, 105). This is in contrast to the essential role of *Tinman* in early specification of the cardiac lineage in *Drosophila* and this can be explained possibly because other NK2 homeodomain proteins overlap, or other cardiac transcription factors can substitute for an early function of Nkx2.5 during heart development in mice (60).

Gain-of-function studies showed the important role of Nkx2.5 in the cardiogenic program. Over-expression of Nkx2.5 in *Xenopus* and zebrafish embryos results in increased heart size and induction of cardiac genes (106, 107). When a dominant-negative Nkx2.5 mutant protein is expressed in frog embryos, the cardiogenesis is blocked; whereas combined expression of both Nkx2.5 and Nkx2.3 dominant-negative mutants results in a more severe phenotype than each mutant does alone (108). These results suggest that a dominant negative mutant protein could be more severe than a gene deletion because it disrupts the regulatory transcriptional control, which is known to be important during heart development.

Nkx2.5 also functions within a complex network of transcription factors controlling heart formation. Nkx2.5 controls the spatiotemporal switch between proliferation and differentiation states of secondary heart progenitor cells and works in a negative feedback loop to inhibit Bmp2/Smad1 signaling (109). Nkx2.5 also transactivates the Ets-related protein 71 (Etsrp71) gene, which is important for endothelial/endocardial fate in the developing embryo. Estrp71 is a direct activator of the endothelial Tie2 gene, which is an upstream of endocardial formation (110).

Nkx2.5 is critical not only during embryogenesis but also after birth. The role of Nkx2.5 has been studied beyond E10.5 in mice, wherein the tamoxifen inducible Nkx2.5 gene knockout mouse model was studied at different stages (E12.5, E19.5, and 2 week old mice) showed morphogenetic cardiac and conduction defects (111–113). Moreover, studies also showed that atrial restricted deletion of Nkx2.5 resulted in cardiac abnormalities including atrial septal defect and conduction system defects (114). However, restricted deletion of Nkx2.5 in the ventricle did not cause cardiac defects but resulted in conduction defects including atrioventricular block (115).

1.1.3.2 GATA4 transcription factor

The GATA4 transcription factor is a member of the zinc finger family of transcriptional regulators. The mammalian genome has six GATA factors. Among them, GATA4, GATA5 and GATA6 are expressed very early during development at E7-7.5 in the pre-cardiac mesoderm and are critical for various stages of

normal heart morphogenesis (116–119). The C-terminal zinc finger is the major DNA binding domain, whereas the N-terminal zinc finger binds DNA with lower affinity. Both C and N-terminal are important for protein-protein interactions and the sequence flanking these region are important for transcriptional activation (120–122).

GATA4 is among the most studied GATA factors in the heart, especially because mutations in this gene are associated with congenital heart defects (CHDs) in humans (123, 124). Several mouse models have been generated to replicate the phenotypes found in human patients (125–127). GATA4 null mouse embryo die at E9.0-10.0 and exhibit cardiac defects including cardiac bifida, looping and septation defects, hypoplastic and poorly trabeculated ventricles (128, 129). Ablation of GATA4 in cardiomyocytes using Nkx2.5^{Cre} causes lethality at E11.5. Hypoplasia of the right ventricle was evident by reduced cardiomyocyte proliferation and the mutant embryo showed an absence of EMT during cushion development (130). However ablation of GATA4 using alpha MHC^{Cre} resulted in lethality by E14.5 with normal EMT but again reduced proliferation and abnormal outflow tract septation (130). These results indicate the stage-specific roles of GATA4 in the formation of right ventricle and OFT, as well as its important during cardiomyocyte proliferation.

During early heart development, combinatorial interaction between transcription factors and GATA4 are important to regulate genes required for cell specification and differentiation (131). For example interaction of GATA4 and Nkx2.5 has a positive transcriptional synergy on several genes important for

cardiac muscle differentiation and chamber formation such as ANF, Cardiac ACTC1, alpha myosin heavy chain (α-MyHC) and Ankyrin Repeat Domain 1 (Ankrd1) (71, 83, 132–134). GATA4 does not only interact with Nkx2.5 but also modulates its activity through a positive feedback loop with SMAD proteins (89). GATA4 can also interact with TBX5 and the multi zinc-finger protein friend of GATA (FOG2) (135, 136). GATA4 can be substituted by other GATA factors such as GATA5 (71).

1.1.3.4 Tbx5 transcription factor

Tbx5 is a member of T-box proteins, which are a large family of conserved transcription factors. Their T-box DNA binding domain contains a highly conserved 180-amino acid. Several members of the T-box family of transcription factors such as Tbx1, Tbx2, Tbx3, Tbx5, Tbx18 and Tbx20, play important roles during heart development (137–139).

Tbx5 is the most studied T-box transcription factor and the first one identified as playing an important role during heart development. In humans, heterozygous mutations in the T-box factor gene Tbx5 cause Holt Oram Syndrome that is characterized by upper forelimb defects and CHDs (140, 141). Tbx5 is expressed early during heart development at the cardiac crescent stage and in developing forelimbs (142). Homozygous Tbx5 null mutation results in embryonic lethality by E10.5 and shows defect during heart looping, underdeveloped atria, EC and severe hypoplasia of the sinoatrial node and left ventricle. Heterozygous null mutation results in limb and cardiac disorders similar

to those observed in human patients (84, 143). Tbx5 is also required for proper development of the conduction system through its interaction with Id2 and Nkx2.5 to ensure proper specification of ventricular myocytes into ventricular conduction lineage (144). The loss of Tbx5 in the mouse ventricle results in embryonic lethality by E11.5 and a single chamber indicating the requirement for Tbx5 in septation (145).Tbx5 physically interacts with Nkx2.5 to promote ANF expression, which is essential for chamber formation in the developing heart (72, 84, 146)

1.1.3.5 Mef2c transcription factor

MEF2C is a member of the MADS-box proteins that are conserved throughout evolution and are critical for regulation of gene expression in skeletal muscles and heart (60, 147). MEF2 genes activate or repress gene transcription via interactions with numerous cofactors (148). There are four MEF2 genes in vertebrate A, B, C, and D, and they share a homology MADS-box in the N-terminus and an adjacent motif known as the MEF2 domain. These two domains are important to mediate DNA binding, homo and heterodimerization, and interaction with basic helix-loop-helix (bHLH) transcription factors (149, 150). The C-terminal regions of MEF2 proteins are highly divergent and also highly variable within a single gene (151).

Of the four vertebrate MEF2 genes, Mef2c has been shown to be a key regulator of cardiac development. Mef2c is required for activation of a subset of cardiac contractile protein genes, as well as for the development of cardiac

structures derived from the SHF and essential for right ventricular development (152). Mef2c homozygous embryos die at E10.5, and the heart tube does not undergo looping and the right ventricle is absent. *dHAND* expression is downregulated in the Mef2c null hearts, which correlates with the absence of the right ventricular. Thus, Mef2c is an essential regulator of cardiac morphogenesis and right ventricular development by regulating *dHAND* expression (152, 153). Mef2c forms a transcriptional network and interacts with several transcription factors during heart development. For example, it interacts with Nkx2.5 *in vivo*, and the compound mutant embryos of $Nkx2.5^{-/-}$ and $Mef2c^{-/-}$ shows more severe cardiac phenotype than those associated with either single mutant, suggesting the requirement of early genetic interaction of Nkx2.5 and Mef2c during heart development (80). Mef2c is also required for proper addition of the FHF precursors to the left ventricle which is important for ventricle cardiomyocyte differentiation (154).

1.2 Congenital heart defects

Congenital heart defects (CHDs) are errors that occur during heart development and they could be 1) morphological abnormalities, resulting in structural malformations; or 2) functional conduction abnormalities, including arrhythmias and cardiomyopathies (31). Defects in heart formation have been detected in ~1% of live births and are a significant cause of morbidity and mortality in newborns. Defects in the cardiac conduction system present a significant mortality risk throughout life (31, 155, 156). CHD results from multifactorial caused including genetic defects or environmental factors (157).

Septum defect including atrial or ventricular defects are one of the most cardiac defects found at birth in patients with mutations in core cardiac transcription factors and it could be atrial or ventricular septum defects. Atrial septal defects (ASD) affect around 1/1000 live births and account for 10% of CHD (158). Insufficient growth of the septum secundum, which is located in the fossa ovalis of the atrial septum after birth, results in atrial septum secundum defect. In patients with ASD, persistent left to right blood shunt may result in atrial and ventricular dysfunctions (157). However, ventricular septal defects (VSD) account for around 40% of all CHDs, therefore, it is the most common congenital heart abnormality. VSDs result from an opening in the interventricular septum (IVS) (158, 159). Another septum formation in the heart is the atrioventricular septal defect (AVSD), which is a defect that occurs during AVC cushion formation and results in an opening between both atria and both ventricles (160). AVSD mostly occurs in patients with trisomy 21 (Down's Syndrome) and accounts for around 5% of all CHDs (160).

1.2.1 Transcription factors associated with congenital heart defects

The molecular mechanisms of congenital heart defects are still not clear. However, genetic studies of families with CHDs showed that mutations in key cardiac transcription factors such as *NKX2.5, GATA4-6, TBX1-5* and others (see Figure 1.3) are major contributors to many forms of cardiac anomalies (161–164). CHDs caused by dominant mutation form of these transcription factors could be a result of loss of function of the protein by interrupting DNA interaction and tightly regulated gene expression profiles, or a result of inactivating one

allele. The molecular and morphological events of heart formation are sensitive to genetic changes (18, 60, 161, 162, 165). Many studies have shown that animal models lacking any of these cardiac transcription factors exhibit severe defects in heart development.

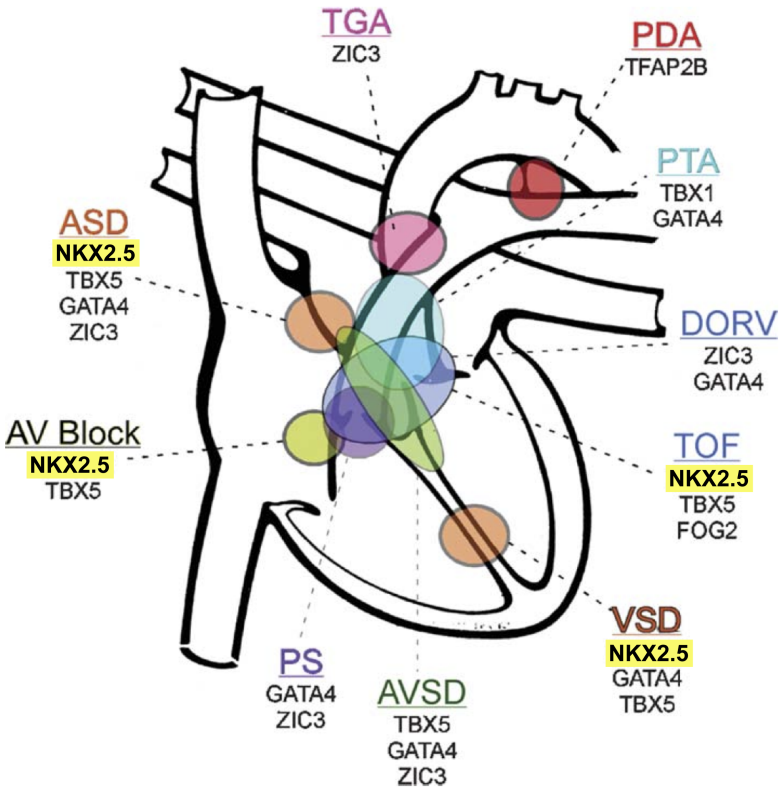


Figure 1.3 Full-chambered heart with sites of congenital heart defects.

Transcription factors shown in the figure are found to be associated with CHDs. (ASD) atrial septal defect; (AV block) atrioventricular block; (AVSD) atrioventricular septal defect; (DORV) double- outlet right ventricle; (PDA) patent ductus arteriosus; (PTA) persistent truncus arteriosus; (PS) pulmonary stenosis; (TGA) transposition of the great arteries; (TOF) tetralogy of Fallot; (VSD) ventricular septal defect. The figure is obtained from (166).

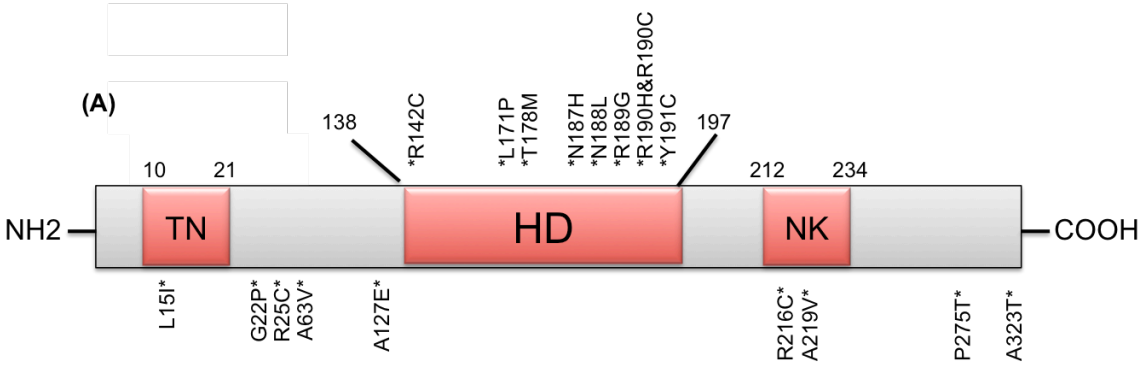
1.2.2 Nkx2.5 mutations and congenital heart defects

In 1998, Nkx2.5 was the first cardiac transcription factor identified as a relevant disease gene for human ASD and atrioventricular (AV) block (167). To date, more than 40 heterozygous mutations in NKX2.5 have been identified in patients who mostly display ASD and AV conduction block. Other cardiac defects include Tetralogy of Fallot (TOF), VSD, Ebstein's anomaly, DORV and hypoplastic left heart syndrome (168–171) (see Figure 1.4). Conduction abnormalities are also associated with mutations in Nkx2.5 including AV block, arrhythmias and increased risk of sudden death (172). In patients with NKX2.5 mutation, there is an association between CHDs and AV block. AV block occurs due to a malformation in the AV node that causes a conduction delay, which could progress during postnatal life and advance into second or third-degree AV block. However, AV block could also develop in patients with heterozygous mutation in NKX2.5 without association with CHDs (173, 174)

Several studies showed that Nkx2.5 heterozygous knockout mice exhibit different heart abnormalities, such as septal and conduction system defects and the penetrance of the disease was influenced by the mouse strain (104, 175, 176). Mice carrying one Nkx2.5-null allele and one Nkx2.5-IRES-cre allele, which produced ~50% less Nkx2.5 protein than the wild type allele, died postnatally, showing a spectrum of cardiac malformations overlapping the more severe defects observed in humans with *NKX2.5* mutations (109). Transgenic mice carrying the I183P mutant Nkx2.5 protein driven by the ß-myosin heavy chain

promoter were born apparently normal but showed progressive and profound cardiac conduction defects and heart failure (177). Also, several conditional knockout models showed that Nkx2.5 function is critical after the mid-embryonic stage and in the perinatal heart, due to the regulation of important gene products involved in conduction and contraction (111, 112). Recently, a heterozygous mouse model of a human CHD mutation in Nkx2.5 (R52G) resulted in pleotropic cardiac anomalies and conduction defects with higher penetrance, whereas the homozygous mice for this mutation were never born (178, 179). Deletion of Nkx2.5 in mesoderm using $Mesp1^{Cre}$ resulted in cardiac defects similar to the one observed in Nkx2.5 null embryos' hearts, and that re-expression of Nkx2.5 in the mesoderm rescued the cardiac defects. In contrast, endodermal deletion of Nkx2.5 using $Foxa2^{cre}$, resulted in normal heart development and mice survived to adulthood. These results highlight the importance of Nkx2.5 expression in the mesoderm during early heart development (180).

A recent study by Bouveret et al. (2015) highlighted an important finding that suggested that Nkx2.5 mutant protein can still interact with co-factors and direct them to usual Nkx2.5 targets. At the same time, these co-factors can direct the mutant protein to abnormal gene targets, "off target genes", leading to congenital heart defects (181, 182).



(B)

Point mutation	Phenotype	Ref
Lys15Ile	ASD	(183)
Glu22Pro	TOF	(184)
Arg25Cys	VSD, TOF	(174, 185)
Ala63Val	L-TGA	(184)
Ala127Glu	ASD	(184)
Arg142Cys	ASD, VSD, AVB, TOF	(186)
Gln170ter	ASD, AVB	(167, 170)
Thr178Met	ASD, AVB, TOF	(170, 187)
Gln187Ter	VSD	(185)
Asn188Lys	ASD, AVB	(174)
Arg189Gly	ASD, AVB	(174)
Arg190Cys	ASD	(170)
Tyr191Cys	ASD, VSD, AVB	(174)
Arg216Cys	TOF	(188)
Ala219Val	TOF	(184, 188)
Pro275Thr	CoA	(187)
Ala323Thr	TOF	(184)

Figure 1.4 Nkx2.5 protein and examples of mutations found in human patients with CHDs.

(A) Localization of point mutations in Nkx2.5 protein found to be associated with CHD in human. **(B)** The point mutations in Nkx2.5 and the phenotypes associated with each mutation. (ASD) atrial septal defect, (VSD) ventricle septal defect, (TOF) tetralogy of fallot, (AVB) atrioventricular block, (L-TGA) transposition of the great arteries, (CoA) coarctation of aorta. The Figure is modified from reference (166).

1.3 Mouse embryonic stem cells and embryonal carcinoma cells

Embryonic stem cells (ESc) and embryonal carcinoma cells are pluripotent cells that are derived from the inner cell mass of a mouse embryo's blastocyst (189–191). Mouse Esc (mESCs) and embryonal carcinoma cells were first isolated in 1981 and 1982, respectively and they are capable of differentiation into neurons, cardiac muscles and skeletal muscles (190, 192–194). Stem cells maintain their pluripotency *in vitro* by expressing pluripotent transcription factors such as Nanog, Oct4 and Sox2. They are also capable of differentiation into multiple cell types, ectoderm, endoderm and mesoderm and they have been used extensively for studying the molecular mechanism of cardiomyogenesis (195, 196). P19 is an example of embryonal carcinoma cells, are derived from a primary teratocarcinoma induced in a CH3/HC mouse (197, 198).

1.3.1 In vitro cardiac differentiation

Under particular conditions, mESCs can be differentiated into spontaneously beating cardiomyocytes (189, 199, 200, 196). *In vitro* cultivation of the ES cells as a hanging drop in a three dimensional (3D) aggregate called embryonic bodies (EB) results in differentiation into three germ layers including cardiomyocytes (201, 195). However, there is a heterogeneous population of cardiomyocytes cells due to the presence of different cell types including atrial, ventricle and conductive cells (202). During mESCs differentiation, signaling pathways and transcriptional gene networks are expressed which mimics the early development of murine embryo. Therefore, researchers can use this *in vitro* model system to study early cardiomyogenesis (Figure 1.5).

1.3.2 Signaling pathway and transcriptional regulation during cardiomyogenesis *in vitro*

The first step of cardiac muscle differentiation is the induction of mesodermal lineages expressing Brachyury (T), which will then lead to the formation of Mesp1 cardiac progenitor cells. These cardiac progenitors express key cardiac transcription factors such as NKX2.5, GATA4 and TBX5, leading to the specification and formation of contractile cardiac muscles (189, 200, 203) (Figure 1.5).

During cardiomyogenesis in mESCs, Wnt signaling plays a bi-phasic role. At early stages of cardiomyogenesis, activation of Wnt/ β -catenin signaling is necessary to promote the induction of cardiac progenitor cells that express

Mesp1. In contrast, Wnt/ β -catenin is inhibitory at late stages of cardiomyogenesis, which is similar to the embryo (204).

Recent work in our laboratory has identified a novel interaction between Nkx2.5 and the myosin phosphatase (MP) enzyme complex during cardiomyogenesis in P19 and mESCs (205). The phosphatase 1β (PP1) is the catalytic subunit, and myosin phosphate (Mypt1) is the targeting subunit 1 of the MP enzyme complex. Myosin phosphatase modulates the balance of phosphorylation of the regulatory light chain of myosin II with myosin light chain kinase, thereby regulating muscle contraction. (206). The study showed that Nkx2.5 interacts specifically with the phosphorylated form of MP, which results in exclusion of Nkx2.5 from the nucleus to the perinuclear region, and a decrease of Nkx2.5 transcriptional activity in P19 cells. Wnt3a, is an inhibitor of terminal cardiomyogenesis and it is known to modulate ROCK (39, 207). When mESCs was treated with Wnt3a at a later stage of cardiac differentiation, it resulted in nuclear exclusion of Nkx2.5 and its co-localization with pMypt1T853 as well as downregulation of cardiac genes expression and reduced cardiomyogenesis. These results suggest that Wnt3a activates ROCK, which phosphorylates Mypt1 at Thr853, resulting in the movement of MP complex to the nucleus where it interacts with Nkx2.5. Subsequently, the MP/Nkx2.5 complex was exported to the perinuclear region, which was accompanied with reduce cardiomyogenesis (205).

BMP signaling is a positive regulator of cardiomyogenesis (208). For example, treatment of mESCs with BMP2 results in increased Nkx2.5 expression

and cardiac structural proteins that lead to increased cardiomyogenesis (209, 210). In contrast, overexpression of the BMP antagonist Noggin in P19CL6 cells inhibited cardiomyogenesis. However, the defect of cardiac differentiation was rescued when BMP protein was added to P19CL6 noggin cells. Also, overexpression of both Nkx2.5 and GATA4 in P19CL6 noggin cells rescued the defect of cardiac differentiation, whereas overexpression of each of these factors alone did not rescue the defect. Thus cross talk or interaction between transcription factors and signaling pathways are important for proper cardiomyogenesis (211).

Several key cardiac transcription factors, such as Nkx2.5, Mef2c and GATA4, can induce cardiomyogenesis in pluripotent P19 cells (212, 213), whereas the dominant-negative of these proteins results in ablation of cardiomyogenesis (212, 214, 215). For example, expression of a dominant negative form of Nkx2.5 in P19 cells resulted in inhibition of cardiomyogenesis in this system, indicating that Nkx2.5 activity is required for this process (216). Moreover, overexpression of Nkx2.5 resulted in enhance cardiomyogenesis in P19 cells (217). A recent study (218) showed that haploinsufficiency of $Nkx2.5^{+/EGFP}$ mESCs in cardiac progenitor cells has a functional defect at early stage of cardiomyogenesis in EBs, where it resulted in delay and reduce cardiomyogenesis when compared to control $Nkx2.5^{+/+}$ mESCs. However, overexpression of Desmin in $Nkx2.5^{+/EGFP}$ mESCs rescued Nkx2.5-related haploinsufficient phenotype (218). Therefore, Desmin regulates Nkx2.5 expression during cardiomyogenesis in mESCs.

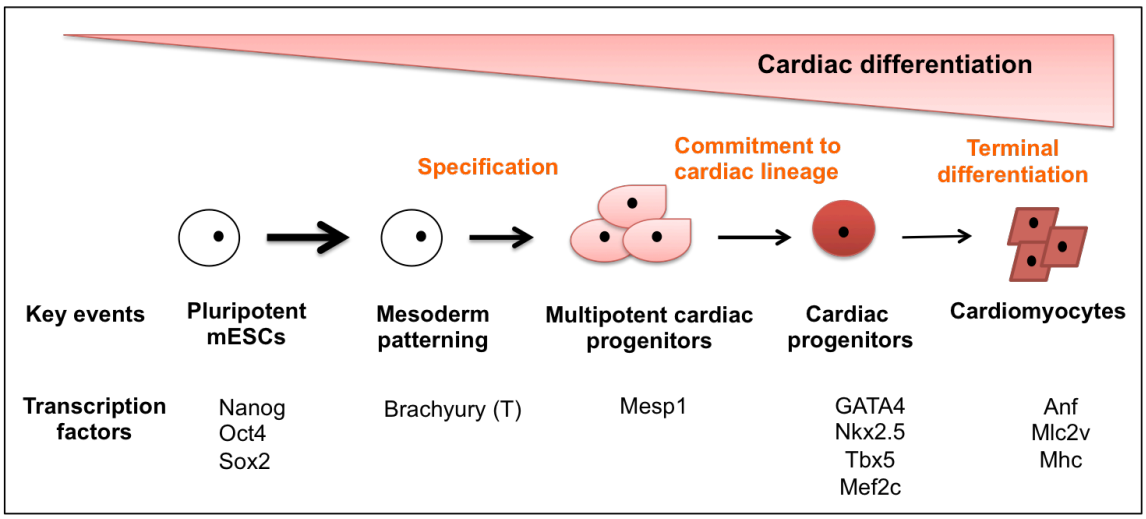


Figure 1.5 Cardiomyogenesis in mouse embryonic stem cells (mESCs).

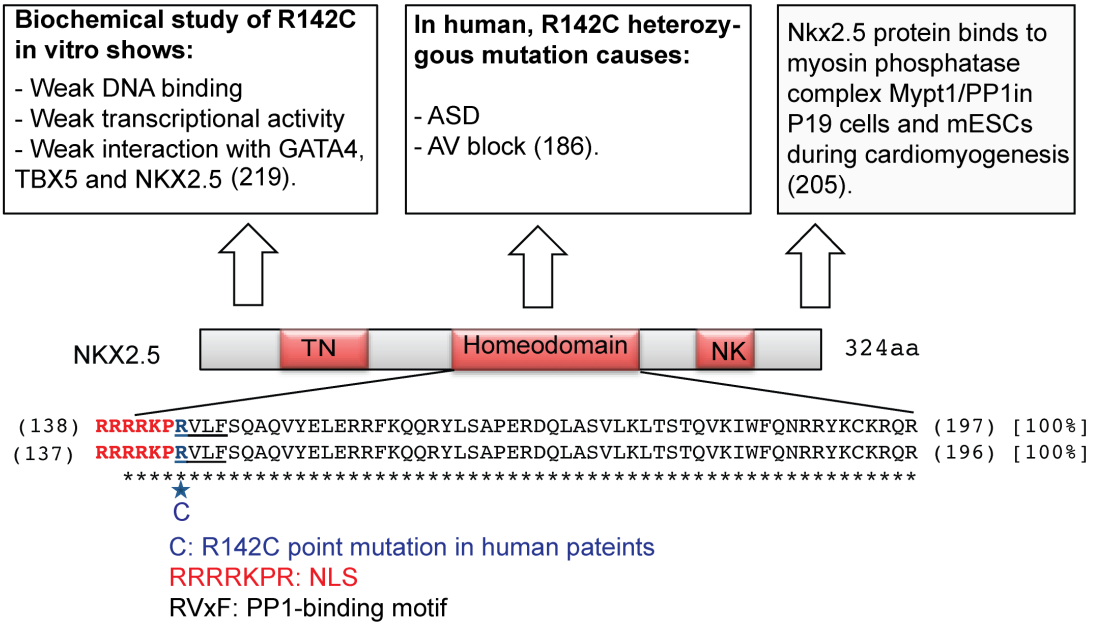
Key events and transcription factors that are involved in the formation of cardiac muscles from pluripotent mESCs. At day 0, mESCs express pluripotent markers that keep them in undifferentiated state such as Nanog, Oct4 and Sox2. Upon differentiation, embryonic bodies undergo mesodermal induction and express Mesp1 and Brachyury (T). Subsequently, the multipotent pre-cardiac mesodermal cells are committed to become cardiac progenitors and start expressing high levels of core cardiac transcription factors such as Nkx2.5, GATA4, Tbx5 and Mef2c. The cardiac progenitors then undergo terminal differentiation resulting in the formation cardiac muscles expressing Mlc2v, ANF and Mhc.

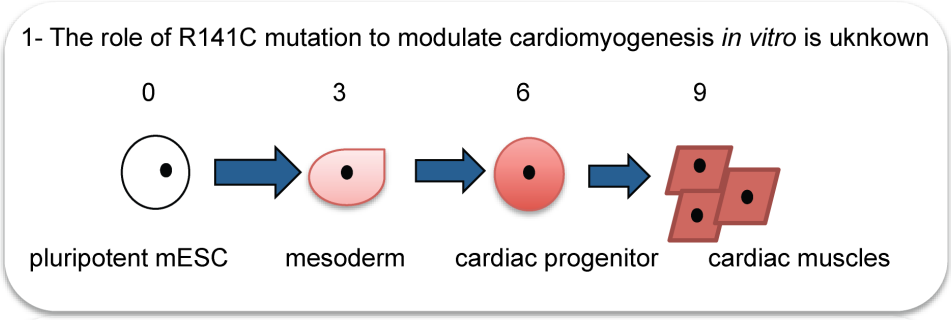
1.4. Thesis Summary

1.4.1 Rationale

While it is clear that Nkx2.5 regulates many aspects of heart development, but the mechanism underlying CHDs is still poorly understood. In my study, I focused on characterizing a human mutation in the *NKX2.5* gene, *R142C*, which was identified in 13 members from one family, all suffering from CHDs (186). The *R142C* point mutation substitutes a conserved arginine 'R' with cysteine 'C' and is located in codon 142 in human's NKX2.5 protein. The reported *in vitro* analysis of the *R142C* mutation showed that the mutant NKX2.5 protein had low DNA binding affinity, low transcriptional activity and weak interactions with other cardiac transcription factors such as GATA4, TBX5, and with itself, when compared to WT Nkx2.5 (219). However, the *R142C* mutation's ability to modulate cardiomyogenesis *in vitro* and to cause CHDs in a mouse model has not been tested yet. Also, when my PhD thesis was initiated there was no mouse model harboring a human causal mutation in Nkx2.5 gene.

A previous study conducted by a colleague at Dr. Skerjanc laboratory (205) showed a novel interaction between Nkx2.5 and the myosin phosphatase complex (MP) subunits Mypt1 and PP1. We found that Nkx2.5 contains a known, conserved PP1 binding sequence, RVLF (220), at the amino terminal region of the Nkx2.5 protein. Interestingly, in patients with the *R142C* point mutation, the conserved "R" in the putative PP1 binding sequence is mutated to "C". However, the *R142C* mutation's ability to bind to MP complex has not yet been investigated (see Figure 1.6).


Biochemical study of R142C in vitro shows:
- Weak DNA binding
- Weak transcriptional activity
- Weak interaction with GATA4, TBX5 and NKX2.5 (219).
In human, R142C heterozygous mutation causes:
- ASD
- AV block (186).
Nkx2.5 protein binds to myosin phosphatase complex Mypt1/PP1in P19 cells and mESCs during cardiomyogenesis (205).
NKX2.5
TN
Homeodomain
NK
324aa
(138) RRRRKPRVLFSQAQVYELERRFKQQRYLSAPERDQLASVLKLTSTQVKIWFQNRRYKCKRQR (197) [100%]
(137) RRRRKPRVLFSQAQVYELERRFKQQRYLSAPERDQLASVLKLTSTQVKIWFQNRRYKCKRQR (196) [100%]
C
C: R142C point mutation in human pateints
RRRRKPR: NLS
RVxF: PP1-binding motif


1- The role of R141C mutation to modulate cardiomyogenesis *in vitro* is uknkown
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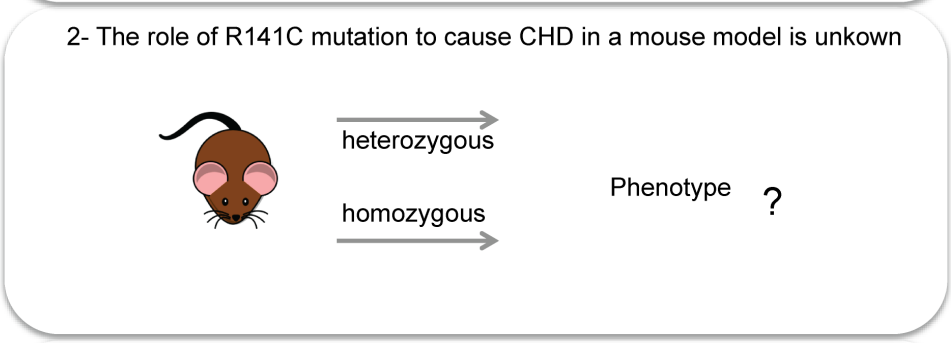
3
6
9
pluripotent mESC
mesoderm
cardiac progenitor
cardiac muscles


2- The role of R141C mutation to cause CHD in a mouse model is unkown
heterozygous
homozygous
Phenotype
?

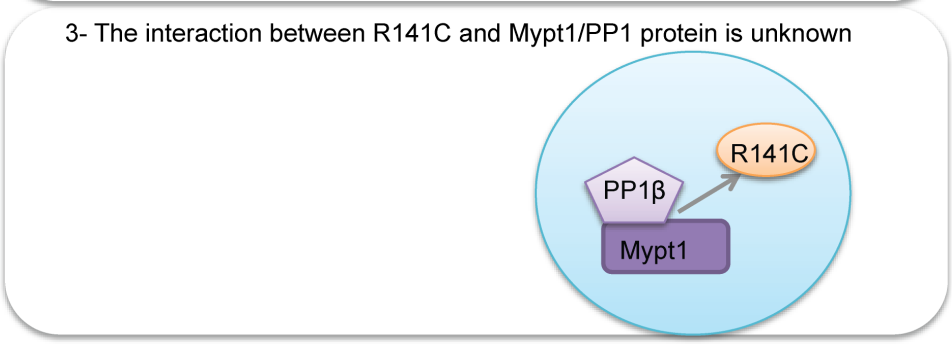

3- The interaction between R141C and Mypt1/PP1 protein is unknown
R141C
PP1β
Mypt1

Figure 1.6 The Rationale for my PhD study.

The open arrows represent previously published work (186, 205, 219). Nkx2.5 protein and the homeodomain sequence are shown. The (NLS) nuclear localization sequence is shown in (Red). The point mutation (Blue) results in the substitution of arginine with cysteine, which is located in the amino acid 142 in human and 141 in mouse. The underlined RVLF sequence is a putative PP1-binding motif (RVxF). The rationales for my PhD study are shown in the boxes under the Nkx2.5 sequence.

1.4.2 Hypothesis and Objectives

1- We hypothesized that the *in vitro* cardiomyogenesis of mESCs, as well as the *in vivo* mouse model harboring the disease-causing mutation (*R142C*), will provide a valuable model for understanding the molecular mechanisms causing the CHDs

2- We also hypothesized that the WT Nkx2.5 interacts with MP complex through the RVxF motif and that the point mutation *R142C* will not interact with the MP complex.

The main aims of my doctoral research are to investigate the role of the *R142C* mutation during cardiomyogenesis *in vitro* and *in vivo* during heart development. Moreover, test the ability of the *R142C* mutation to interact with the MP complex. The objectives of my study are:

1- To generate a heterozygous knock-in mESC that harbors the human point mutation *R142C* in the murine Nkx2.5 gene and examine the ability of the mutation to modulate cardiomyogenesis *in vitro*.

2- To generate and characterize the mouse model harboring the human point mutation *R142C* in the murine *Nkx2.5* gene and examine the ability of the mutation to cause CHDs *in vivo*.

3- To study the interaction of the R142C mutant protein with MP complex *in vitro*.

1.4.3 Summary of the results

Chapter 2 describes experiments designed to test the ability of Nkx2.5 R141C to modulate cardiomyogenesis *in vitro* using a knock-in mESC (*Nkx2.5*$^{R141C/+}$). Of note, the mutation that substitutes the arginine with a cysteine is located in codon 141 in the murine Nkx2.5. We show that the *Nkx2.5*$^{R141C/+}$ mESC has reduced cardiomyogenesis compared to control. We also show that Nkx2.5 protein is aberrantly distributed between the nucleus and cytoplasm during cardiomyogenesis in *Nkx2.5*$^{R141C/+}$ mESC. Finally, gene expression profiling shows misregulation of several genes implicated in cardiac development and function in *Nkx2.5*$^{R141C/+}$ compared to *Nkx2.5*$^{+/+}$ cells, during the early stages (day 6) of the *in vitro* cardiomyogenesis.

Chapter 3 characterizes the mouse model harboring the human mutation *NKX2.5 R142C*. We show that *Nkx2.5*$^{R141C/R141C}$ homozygous embryos die between E9.5 and E10.5 with delayed heart morphogenesis compared to control. We also show that *Nkx2.5*$^{R141C/+}$ heterozygous mice are fertile and normal. However, histological examinations of the newborns and gross morphological examination of the adult mice show that the *Nkx2.5*$^{R141C/+}$ hearts display septum cardiac defects. Finally, the functional studies using ECG show that *Nkx2.5*$^{R141C/+}$ adult mice display AV block with high penetrance compared to their wild type littermates. Therefore, the *Nkx2.5*$^{R141C/+}$ mice serve as a good model for studying human CHDs.

Based on the previous finding that Nkx2.5 interacts with Mypt1/PP1 (205), we examined whether the R141C that has the point mutation in the PP1-binding motif would still bind to Mypt1/PP1. In Chapter 4, experiments were designed to examine the transcriptional activity and localization of both WT Nkx2.5 and R141C protein in the presence or absence of Mypt1/PP1 complex, as well as to study protein- protein interaction between R141C and Mypt1/PP1 complex. We show that the R141C mutant protein still interacts with Mypt1/PP1 but is resistant to Mypt1/PP1 mediated inhibition of R141C transcriptional activity and exclusion to perinuclear region.

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CHAPTER 2

Altered Cardiomyogenesis in Mouse Embryonic Stem Cells Expressing a human *NKX2.5* Missense Mutation Associated with Congenital Heart Defects

Abeer F. Zakariyah[1], Rashida F. Rajgara[1], Ellias Horner[1, 2, 3], Marie-Elodie Cattin[6], Alexandre Blais[1, 2, 3*], Ilona S. Skerjanc[1*] and Patrick G. Burgon[3, 4, 5, 6*]

[1]*Department of Biochemistry, Microbiology, and Immunology, University of Ottawa, Ottawa, Ontario K1H 8M5, Canada.*[2]*Ottawa Institute of Systems Biology, Department of Biochemistry, Microbiology and Immunology, Faculty of Medicine, University of Ottawa, 451, Smyth Road, Ottawa, Ontario, K1H 8M5, Canada.*[3]*University of Ottawa Center for Neuromuscular Disease.*[4]*University of Ottawa Heart Institute, 40 Ruskin Street, Ottawa, Ontario, K1Y 4W7, Canada.*[5]*Department of Cellular and Molecular Medicine, University of Ottawa, Ottawa, Ontario K1H 8M5, Canada*[6]*Department of Medicine (Division of Cardiology), University of Ottawa, Ottawa, Ontario K1H 8M5, Canada*

To whom correspondence should be addressed: Prof. Patrick G. Burgon, Email: pburgon@ottawaheart.ca; Prof. I.S. Skerjanc, Email: iskerjanc@uottawa.ca; Prof. A. Blais, Email: alexandre.blais@uottawa.ca

My contributions to the chapter are the following:

In this manuscript, I performed all the reported experiments with the exception of the microarray and RT- q PCR in (Figure 2.6). Elias Horner performed the microarray experiment and Dr. Alexandre Blais analyzed the data. Rashida F. Rajgara performed the RT- q PCR in (Figure 2.6). Marie-Elodie Cattin helped performing the reporter assay in (Figure 2.5). The targeting construct (Fig 2.1 B) was designed and created by inGenious targeting laboratory. I wrote the manuscript with the contribution of Dr. Alexandre Blais and Patrick G. Burgon.

Currently, this manuscript is under revision

2.1 Abstract

The *Nkx2.5* gene codes for a transcription factor that plays a critical role in heart development; heterozygous mutations in *NKX2.5* in human and mice result in congenital heart defects. However the molecular mechanisms by which these mutations cause the disease are still unknown. In our study, we investigated the effect on cardiomyogenesis of the *Nkx2.5* missense mutation associated with congenital heart defects in human, which affects the nuclear localization signal (NLS). In this report, we created knock-in mouse embryonic stem cells heterozygous for the homologous mutation in the mouse gene ($Nkx2.5^{R141C/+}$) and examined their ability to undergo cardiomyogenesis *in vitro*. We found that heterozygosity for *Nkx2.5 R141C* results in an alteration in the expression of genes essential for normal heart development that is accompanied by reduced *in vitro* cardiomyogenesis. We also demonstrate that subcellular localization of Nkx2.5 protein is disrupted in $Nkx2.5^{R141C/+}$ cells during cardiomyogenesis. Moreover, gene expression profiling of $Nkx2.5^{R141C/+}$ cells revealed, at an early stage of cardiac differentiation (day 6), a global deregulation of gene expression and identified putative direct target genes of *Nkx2.5* that are affected by the R141C mutation. Taken together, our data supports the notion that Nkx2.5 concentration in the nucleus is critical for normal cardiomyogenesis.

2.2 Introduction

Congenital heart defects are the most common genetic abnormalities and affect approximately 1 % of newborns (1). Heart development is a complex multi-step morphogenetic process, initially from the establishment of the cardiac crescent to the formation of the linear heart tube, followed by cardiac looping, chamber formation, septation and valve formation (2). The heart development process is coordinated through the sequential and succinct spatiotemporal control of gene expression, which is mediated by a core set of cardiac transcription factors, including *NKX2.5*, *GATA-4,* and *TBX5*. Patients with congenital heart defects often show a genetic linkage with mutations in one of these transcription factors (1). Nevertheless, the molecular mechanism underlying many congenital heart defects remains poorly understood.

The homeobox transcription factor *Csx/Nkx2.5* (MIM# 600584) is one of the earliest expressed transcription factors in the heart. Detailed analysis of *Nkx2.5's* role in heart development has revealed that *Nkx2.5* controls many aspects of cardiomyogenesis, including cellular specification, proliferation and differentiation (3, 4). Previous studies in mice have demonstrated that the loss of *Nkx2.5* expression results in embryonic lethality, between days E9.5 and E10.5, that is associated with defects in heart looping, myocardial differentiation, as well as the absence of the endocardial cushion (5, 6). Furthermore, heterozygous *Nkx2.5* knockout mice exhibit heart abnormalities, such as septal and conduction system defects (7). Recently, a mouse model of a human congenital heart defect with a non-synonymous substitution in *Nkx2.5 (R52G)* was reported. The

heterozygous *Nkx2.5*$^{R52G/+}$ mice displayed varied cardiac anomalies with high penetrance with embryonic lethality reported in the homozygous *Nkx2.5*$^{R52G/R52G}$ mice (8).

At the molecular level*, Nkx2.5* functions by directly binding to specific DNA elements to regulate a number of cardiac genes (9), as well as synergistically interacting with other cardiac transcription factors, such as *Gata-4*, *Mef2c*, and *Tbx5* to activate or repress the expression of genes critical for cardiac muscle formation and specification (10–12). The homeodomain of Nkx2.5 is required for DNA binding, protein-protein interactions, and also contains a nuclear localization signal (NLS) RRRRKPR, which has been shown to be required for Nkx2.5 localization to the nucleus (13).

In humans, more than 40 *NKX2.5* variants have been described to be associated with congenital heart defects (14). In the current investigation, we focus on a previously reported non-synonymous substitution that gives rise to an autosomal dominant *NKX2.5 R142C* variant that was associated with congenital heart defects in individuals of one family (15). The *NKX2.5 R142C* variant is found in 13 patients from one family, with 12 of 13 displaying atrioventricular block and 9 of 13 presenting atrial septal defect (15, 16). *In vitro* experiments demonstrated that NKX2.5R142C protein has a reduced DNA binding affinity, attenuated transcriptional activity and reduced physical interaction with wild-type NKX2.5, GATA-4, or TBX5 proteins (16). The previous study demonstrated that the R142C substitution that is located within the NLS of NKX2.5 did not disrupt *NKX2.5 R142C* from translocating to the nucleus when overexpressed in COS7

(immortalized monkey fibroblast) cells (13, 16). However, the functional impact of the *NKX2.5 R142C* mutation on cardiomyogenesis remains to be elucidated.

To gain greater insight into the molecular function of *NKX2.5 R142C* variant during cardiomyogenesis, we have generated and characterized heterozygous mouse embryonic stem cells (mESCs), harboring the mutation that substitutes the arginine with a cysteine within codon 141 in the murine *Nkx2.5* gene termed (*Nkx2.5*$^{R141C/+}$) cells. The current study presents data that demonstrates that the R141C allele functions as an autosomal-dominant mutation. When the mESCs heterozygous for the Nkx2.5 R141C mutation are induced to differentiate into cardiomyocytes, the nuclear localization of Nkx2.5 R141C protein is impaired with reduced transcriptional activity, which manifests in the lowering of the expression level of key Nkx2.5 target genes and subsequently reduces the capacity for *in vitro* cardiomyogenesis. To our knowledge, this is the first study using a mESCs harboring a human mutation in the *Nkx2.5* gene.

2.3 Material and methods

2.3.1 Generation of Nkx2.5$^{R141C/+}$ mESCs

A targeting construct (Figure 2.1 A) was designed and created by inGenious Targeting Laboratory (Ronkonkoma, NY). The linearized vector (2 µg) was introduced into 2 x 10^6 trypsinized R1-mESCs by electroporation using 4D-Nucleofector (Lonza, Basel, Switzerland) using the CG-104 program as per the Manufacturer's protocol. Cells were grown in selection medium containing G-418 (200 µg/mL) for 10 days. The homologous recombination event was confirmed by

PCR and by southern blotting using external probes, and DNA sequencing to ensure the presence of the point mutation. Subsequently, Cre recombinase (a generous gift from Dr. Lohnes) was introduced in order to excise the floxed neo cassette. The Cre recombinase expression plasmid was electroporated into positive clones and grown until colonies were observed. DNA was isolated followed by PCR using specific primers to confirm neo excision.

2.3.2 Cell culture and in vitro differentiation

R1 mESCs (a generous gift from Dr. David Lohnes, University of Ottawa) were cultured on mitomycin C-treated mouse embryonic fibroblasts (MEFs) in mESCs culture medium (high glucose (4.5 g/L) DMEM, 15% FBS, 1X MEM non-essential amino acids, 1X Penicillin/Streptomycin, 10 μM β-mercaptoethanol and leukemia inhibitory factor (LIF). Cultures were maintained at 37°C and 5% CO_2, fed every two days and sub-cultured using 0.25% Trypsin-EDTA. Differentiation of ES cells was induced through the formation of embryoid bodies (EBs) as described previously (17). Concisely, EBs were formed in mESC media without LIF in hanging drops containing 800 cells per 20μL droplet on the lid of a petri dish and cultured for two days. EBs were then collected from the droplets and cultured for 3 days in suspension culture in Petri dishes. At day 5, EBs were plated on 0.1% gelatin-coated coverslips for the remaining days of the 9-day differentiation protocol with the medium being changed every day.

2.3.3 Functional cardiomyocyte assessment

At day 5 of EB differentiation, 20-50 EBs were transferred from the Petri

dishes into 6 well plates for RNA and protein extraction or in 12 well plates that had 0.1% gelatin covered coverslips for immunofluorescence analysis. At day 7, 8 and 9, EBs containing beating areas were scored. A total number of at least 30 EBs were counted for each cell line per experiment.

2.3.4 Immunofluorescence

Differentiated EBs on day 7 and 9 were fixed in 4% PFA for 15 min at room temperature, washed with phosphate buffered saline (PBS) and permeabilized with 0.5 % Triton X-100/PBS for 15 min. After three successive washes in PBS, fixed cells were blocked in 0.1% BSA, 10% donkey serum in 0.1% Triton X-100/PBS for 1h at room temperature, then incubated with primary antibodies overnight at 4°C. Cells were then washed three times with PBS for 5 min each and incubated with secondary antibodies (Alexa Fluor 488 donkey anti-goat or Cy3 donkey anti-mouse) at a 1:100 dilution with PBS for 1 h at room temperature. Hoechst dye (Sigma-Aldrich, cat.no.B-2883) was used to counterstain nuclei. The following primary antibodies were used for immunofluorescence: Nkx2.5 (goat; 1:50, Santa Cruz; N-19), MF20 (mouse; 1:1, DSHB), Sarcomeric Alpha-Actinin (mouse; 1:50, Abcam; EA-53). Cells were then visualized using a Leica DMI6000B inverted fluorescent microscope (Leica Microsystems Inc). Cardiac cell differentiation was quantified by manual cell counts of MHC+ cardiomyocytes using the Volocity software (PerkinElmer Inc., Waltham, MA) and represented as a percentage of total nuclei. The error bars represent ± standard error of the mean (SEM) and 5 fields of at least 3 independent experiments.

2.3.5 Western blot analysis

Total protein extracts were collected from cells on days 0, 6, and 9 using RIPA buffer (50mM Tris-HCl, pH 7.4, 1% NP-40, 0.25% Na-deoxycholate, 150mM NaCl, 1mM EDTA) containing 0.5 mM PMSF (Sigma-Aldrich) and 1X complete mini EDTA-free Protease Inhibitor Cocktail (Roche). Nuclear and cytoplasmic extraction was performed as follows. Briefly, on day 9 of differentiation, cell pellets were Dounce homogenized (20 tight pestle-B) in lysis buffer containing 4 mM HEPES, pH 7.4, 320 mM sucrose, 1 mM dithiothreitol, 10 mM $MgCl_2$, 5 mM KCL, 0.1% Triton X-100, protease and phosphatase inhibitors. The homogenate was then centrifuged at *2,000 x g* for 3min at 4°C. The resulted pellet was kept for nuclear extraction. The supernatant was then transferred to new tube and re-centrifuge at *2,000 x g* for 10min at 4°C and saved as cytosolic fraction. Then 5x RIPA buffer was added and centrifuge at *2,800 x g* for 5min. Then the pellet that contains the nuclear fraction was washed 3 times with lysis buffer and centrifuged at *2,000 x g* for 10min at 4°C. Finally, the nuclear pellet was re-suspended in 1x RIPA buffer which was equal to the volume of the cytosolic fraction. An equal volume of the nuclear and cytoplasmic fraction was separated using a 10% sodium dodecyl sulfate polyacrylamide gel. Resolved proteins were transferred to nitrocellulose membrane, which was blocked with 5% non-fat dry milk in TBST (Tris-buffered saline solution with 0.05% v/v Tween-20") and incubated with primary antibodies against NKX2.5 (goat; 1:100, Santa Cruz; N-19), α-Tubulin (1:10.000; DM1A, Sigma-Aldrich), or Nucleolin (a generous gift from Dr. Mona Nemer University of Ottawa, Rabbit; 1:2000; Cell

Signaling). Chemiluminescence was generated using appropriate secondary horseradish peroxidase-conjugated antibodies, anti-mouse (1:5000, Cell Signaling) anti-rabbit (1:5000, Santa Cruz) and anti-goat (1:5000, Santa Cruz), followed by a chemiluminescent reaction using Pierce ECL substrate or SuperSignal™ West Dura Extended Duration Substrate (Fisher Scientific).

2.3.6 RNA isolation and Quantitative Real time PCR

Total RNA was harvested from cells on days 0, 3, 6, and 9 of mESC differentiation using the E.Z.N.A. ® Micro-elute Total RNA Kit I as per manufacturer's instruction (Omega Bio-tek, USA). Purified RNA (500ng) was reverse transcribed into cDNA using QuantiTect Reverse Transcription Kit (Qiagen) as per manufacturer's instruction. To assess successful removal of genomic DNA, a no reverse transcriptase reaction was included. The KAPA SYBR® FAST Universal RT- q PCR Master Mix (KAPA Biosystems) was used as per manufacturer's instruction and performed on the Mastercycler® Realplex qPCR machine (Eppendorf, Canada). Samples were analyzed in duplicate and the average of the two values was used for further analysis. Only samples showing no signal in the no RT reactions were further analyzed. Data were normalized to β-actin and analyzed using the delta-delta CT method to quantify gene expression as described (18), then normalized to *Nkx2.5*$^{+/+}$ day 0 monolayer culture and expressed as percent maximum. The error bars represent ± standard error of the mean (SEM) of at least three independent biological experiments and using three clonal populations per cell line.

2.3.7 Gene expression profiling

RNA was collected on day 0 and 6 from *Nkx2.5*$^{R141C/+}$ or *Nkx2.5*$^{+/+}$ cells (n=2) (pool of 2 colonies on each day per experiment). The quality of the RNA samples was confirmed using Agilent 2100 Bioanalyzer and all samples were profiled using Agilent 8x66 K-Mouse Genome Microarrays (Agilent Technologies, Santa Clara, CA, USA). Raw processed probe intensity data were filtered and quantile normalized using Expander 7.1 (19–21). Briefly, tabular expression data were imported into Expander 7.1 and had a baseline value of 5.0 set for all missing probe expression values. Probes were further filtered by requiring that expression is detectable in at least 2 of the four samples. Expression values were $\log^2$ -transformed, quantile normalized and the probes were merged by Gene ID. Only probes showing at fold-change of expression of at least 2 in both biological replicates were retained.

2.3.8 Cell Culture of HL1 cells, Transfection, and Leptomycin B Treatment

HL1 cells were grown in Claycomb medium as previously described (22) supplemented with 10% fetal bovine serum 2mM l-glutamine 0.1mM norepinephrine and 100 U/ ml penicillin and 100mg/ml streptomycin and incubated at 37°C, in a humidified atmosphere of 5% CO_2. All culture dishes were pre-coated with the gelatin-fibronectin substrate. Twenty-four hours before transfection cells were seeded onto sterile glass coverslips coated with gelatin-fibronectin in a 24-well plate. Transient transfections of WT Nkx2.5 or R141C mutant expression vector were carried out with Lipofectamine 3000 transfection reagent following manufacturer's protocol. Leptomycin B (Sigma) was added to

the culture medium 24 hours after transfection to a final concentration of 20ng/ml for 4 hours.

2.3.9 Reporter gene assay

P19 cells were transiently co-transfected with different amounts (100ng, 200ng, 300ng, 400ng) of Flag tagged Nkx2.5 WT or Flag tagged Nkx2.5 R141C expression plasmid, along with 100ng of ANF-luciferase reporter and 50 ng of SV40-Renilla as an internal control. The Nkx2.5 WT Flag was previously described(17) and the R141C mutants were generated using the QuikChangeII-XL kit, as per the manufacturer's instructions. The ANF reporter has been also previously described (17, 23), SV40-Renilla (Promega). To examine the effects of R141C mutant on transcriptional activation of WT Nkx2.5, the plasmid amount of WT (200ng) and R141C (200ng) was co-transfected together with the plasmids listed above. Total plasmid amount was adjusted to 1μg with empty vector. Transfected cells were harvested after 24h and luciferase assays were conducted as per manufacturer's instructions (Dual-Luciferase® Reporter Assay System, Promega). Samples were analyzed on an LMax II 384 luminometer (Molecular Devices). Firefly luciferase activity was normalized to Renilla and reporter activation was quantified relative to the empty vector control.

2.3.10 Statistical analyses

Statistical analysis was performed using data from at least three independent biological replicates in each cell line. Relative and normalized fold expression values were calculated in Microsoft Excel. The statistical significance

was estimated using Student's t-test. Differences were scored as statistically significant at *p<0.05, **p<0.001 and ***p<0.0005.

2.4 Results

2.4.1 Generation of knock-in mESCs harboring the NKX2.5 R141C non-conservative substitution

The non-conservative substitution, R142C, in the human NKX2.5 allele resides within the NLS that is found at the amino-terminal end of the homeodomain of NKX2.5 (Figure 2.1 A). To define the functional deficits of the *NKX2.5 R142C* variant on cardiomyocyte differentiation, we first generated a knock-in heterozygous mESCs line expressing the R141C substitution in the mouse *Nkx2.5* gene (*Nkx2.5*$^{R141C/+}$) cells. We introduced the genomic fragment that was cloned from 129/Sv mouse strain of the *Nkx2.5* gene into R1 mESCs. The targeting vector was constructed to introduce i) a Flag epitope coding sequence upstream of the *Nkx2.5* coding sequence, before exon 1, ii) the introduction of (CGC to TGC) non-synonymous substitution at codon 141 (located in exon 2), and iii) insertion of a lox FRT-flanked neo cassette within the intergenic region (Figure 2.1 B). Southern Blot analysis confirmed the integration of the targeting vector into the *Nkx2.5* locus (Figure 2.1 C) with the fidelity of targeting assessed by DNA sequencing. Furthermore, to minimize the foreign DNA sequence within the *Nkx2.5* locus, we excised the neo cassette by expressing the Cre recombinase. Then PCR was performed using specific primers to ensure that the neo cassette was excised (Figure 2.1 D). Three

independent clones from *Nkx2.5*$^{R141C/+}$ were compared to their parental R1 mESCs line and WT-Flag Nkx2.5 (*Nkx2.5*$^{+/+}$) cells, in the following analysis. Undifferentiated *Nkx2.5*$^{R141C/+}$ were morphologically indistinguishable to undifferentiated *Nkx2.5*$^{+/+}$ colonies. Moreover, both *Nkx2.5*$^{+/+}$ and *Nkx2.5*$^{R141C/+}$ cells exhibited high mRNA levels of the core pluripotent markers, such as *Oct4* and *Nanog* (Figure 2.6 A). Collectively these data suggest that the *Nkx2.5*$^{R141C/+}$ has no observable alteration in cell pluripotency.

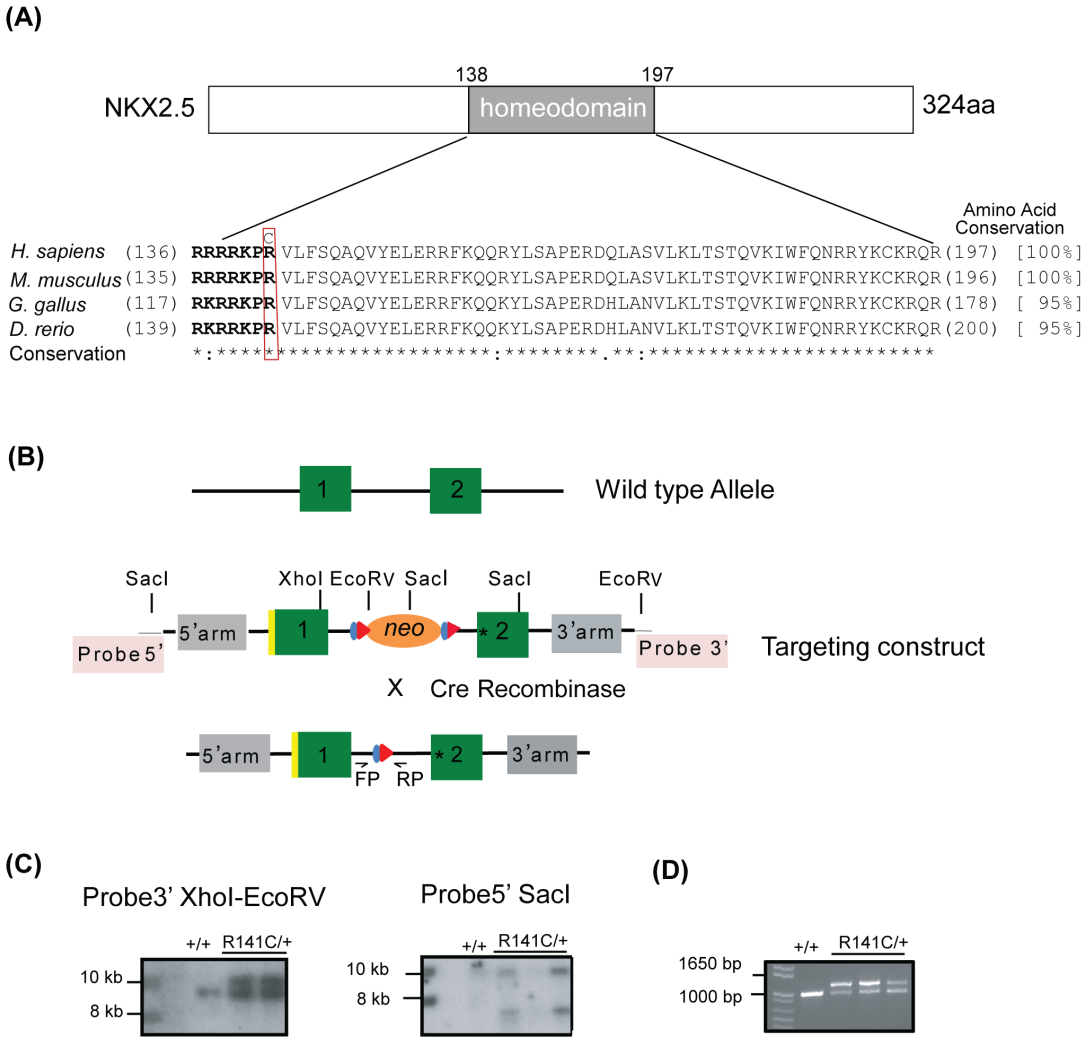


Figure 2.1 Generation of a knock-in mESCs having the human NKX2.5 141 Arginine to Cysteine (R141C) point mutation in the homeodomain at the position of (A) A schematic representation of human NKX2.5 gene with the homeodomain. Nuclear localization sequence (bold) and the substitution of R to C (red box) in patients with congenital heart defect. Primary protein sequence alignment of NKX2.5 homeodomain, below the protein sequence alignment is a key denoting conserved sequence (*), conservative changes (:), and semi-conservative changes (.). Between species NKX2.5 homeodomain amino acid conservation (%) relative to *H. sapiens* listed at the end of each protein sequence. **(B)** Structure of WT *Nkx2.5* locus and the targeting construct electroporated in R1 mESCs. Blue (FRT), red (lox), yellow (flag), asterisk (R141C). **(C)** Identifying positive targeted mESC clones. Southern blotting was performed to identify correctly recombined R141C/+ clones. Unique probes from 3' or 5'outside of homologues arms were used and restriction enzymes (XhoI and EcoRV) or SacI were used to digest the DNA respectively. **(D)** PCR confirming the excision of Cre recombinase.

2.4.2 *Nkx2.5*$^{R141C/+}$ cells display normal mesodermal induction but with lower capacity for cardiac differentiation

mESCs have been used as a unique *in vitro* developmental model for understanding the genetic events regulating the earliest stage of cardiomyogenesis (24, 25). One of the commonly used methods to model the events of early stages of lineage induction and specification of cardiomyogenesis is the formation of embryonic bodies (EBs) (26). To evaluate the capacity of

Nkx2.5$^{R141C/+}$ cells to undergo *in vitro* cardiomyogenesis, we differentiated these cells along with the *Nkx2.5*$^{+/+}$ cells by the formation of EBs for 9 days in culture and measured changes in gene expression during differentiation. EBs typically undergo mesodermal induction by day 3 of differentiation (27). The up-regulation of *Brachyury (T)* is indicative of mesodermal induction (28). Further, *Mesp1* is an early marker for cardiac progenitors that is shown to be elevated before the induction of core cardiac transcription factors (29, 30). Therefore, we examined the mesodermal induction of both *Nkx2.5*$^{+/+}$ and *Nkx2.5*$^{R141C/+}$ cells by measuring the mRNA level of both *Brachyury (T)* and *Mesp1* by RT- q PCR. The RT- q PCR results showed that both *Nkx2.5*$^{+/+}$ and *Nkx2.5*$^{R141C/+}$ cells have elevated *Brachyury (T)* RT- q PCR and *Mesp1* mRNA levels at day 3 compared to day 0, but there was no significant difference in the transcript levels between the two cell lines, suggesting that mesodermal induction, as well as commitment towards the cardiac progenitor stage, occurs normally in both cell lines (Figure 2.2 A).

During cardiomyogenesis, the pre-cardiac mesoderm cells that express *Mesp1* are committed to becoming cardiac progenitors and start expressing high levels of core cardiac transcription factors such as *Nkx2.5, Gata-4, Mef2c*, and *Tbx5* (15, 31). We examined the temporal expression of these genes by RT- q PCR days 6 and 9 of cardiac differentiation. We found that the expression pattern of *Gata-4* and *Nkx2.5* mRNA level in *Nkx2.5*$^{R141C/+}$ cells was comparable to those of *Nkx2.5*$^{+/+}$ cells during differentiation. Moreover, RT- q PCR results revealed that the *Nkx2.5*$^{R141C/+}$ cells showed a significant reduction of *Tbx5* and *Mef2c* mRNA levels on days 6 and 9 when compared to *Nkx2.5*$^{+/+}$ cells, indicating that

Nkx2.5$^{R141C/+}$ cells are defective in up-regulating these two critical cardiac differentiation genes (Figure. 2.2 B).

At later stages, cardiac progenitors terminally differentiate into mature cardiomyocytes and express sarcomeric structural protein and chamber-specific cardiac genes, such as myosin heavy chain (*Myh7*), cardiac alpha-actin (*Actc1*), myosin light chain (*Mlc2v*), and atrial natriuretic factor (*Anf*) (32). We observed that the expression of all of these genes was significantly decreased in the *Nkx2.5*$^{R141C/+}$ cells on days 6 and 9 of differentiation compared to *Nkx2.5*$^{+/+}$ cells (Figure 2.2 C). Taken together, these results reveal that the heterozygous Nkx2.5 R141C mutation does affect the capacity of mESCs to differentiate into cardiomyocytes with no impact on earlier mesoderm commitment.

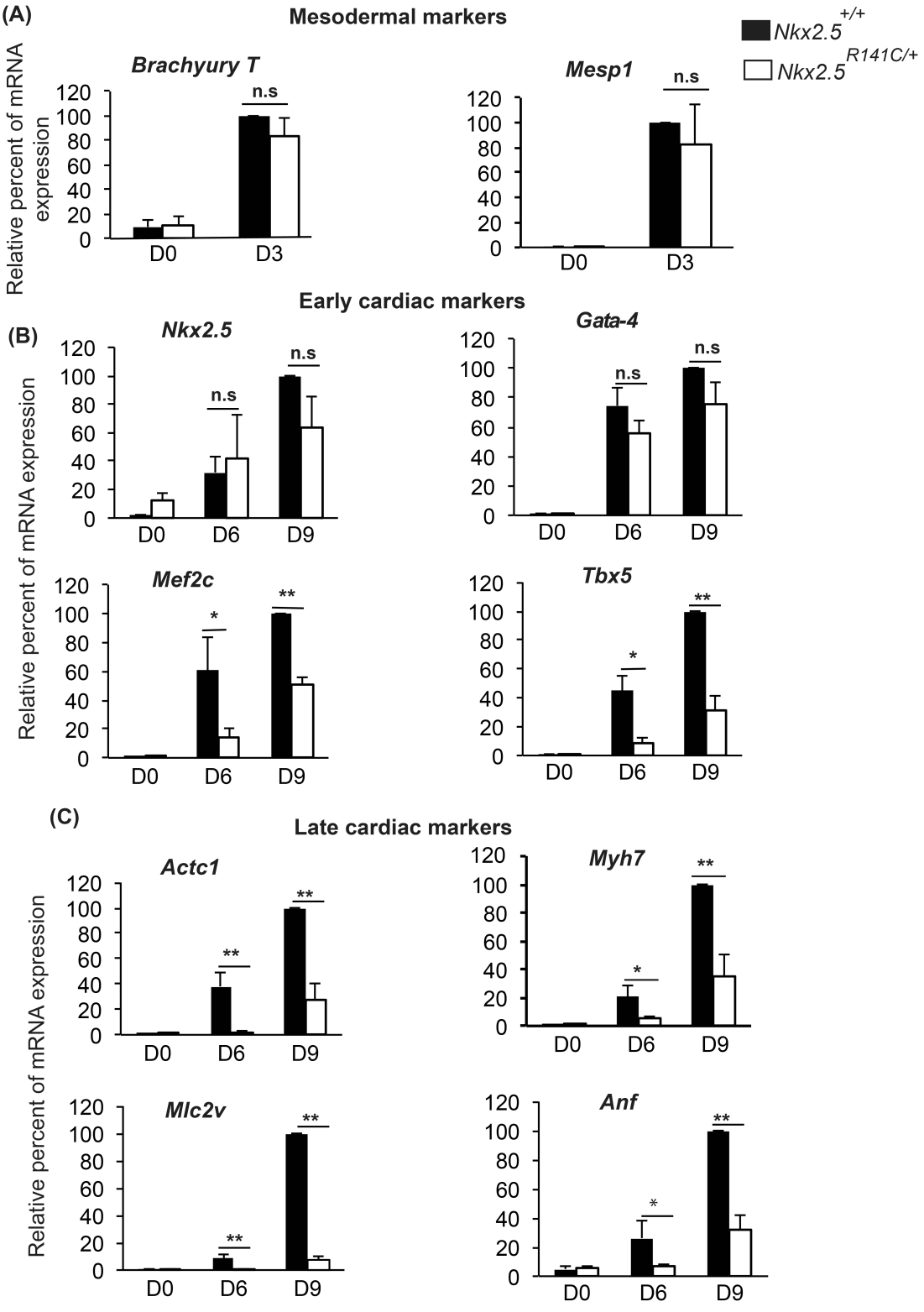

(A)
Mesodermal markers
Nkx2.5+/+
Nkx2.5R141C/+
Brachyury T
Mesp1
Relative percent of mRNA expression
n.s
D0
D3
(B)
Early cardiac markers
Nkx2.5
Gata-4
Mef2c
Tbx5
Relative percent of mRNA expression
n.s
*
**
D0
D6
D9
(C)
Late cardiac markers
Actc1
Myh7
Mlc2v
Anf
Relative percent of mRNA expression
**
*
D0
D6
D9

Figure 2.2 *Nkx2.5$^{R141C/+}$* cells show similar mRNA level of mesodermal markers but altered expression level of some early and late cardiac genes compared to *Nkx2.5$^{+/+}$* cells.

(A) RT- q PCR analysis of mesodermal markers at day 0 and 3 of differentiation **(B, C)** Early and late cardiac genes markers as indicated on days 0, 6 and 9 in *Nkx2.5$^{+/+}$* and *Nkx2.5$^{R141C/+}$* cells respectively. Data is relative to *Nkx2.5$^{+/+}$* day 0 and represented as percent maximum. n.s = not significant, Error bars represent ±SEM, (n=4 *p<0.05 and **p<0.01).

2.4.3 Decreased cardiomyogenesis and cardiac beating in *Nkx2.5$^{R141C/+}$* cells

mESCs can differentiate into spontaneously beating cardiomyocytes. As an indication of cardiomyocyte formation, we monitored the number of spontaneously contracting EBs at days 7, 8 and 9 of differentiation in culture. We observed an increase in the number of contracting EBs from day 7 to day 9 in both *Nkx2.5$^{R141C/+}$* and *Nkx2.5$^{+/+}$* cultures. By day 9 of differentiation, we observed beating in 50% of EBs from *Nkx2.5$^{+/+}$* cells. Instead, only 23% of *Nkx2.5$^{R141C/+}$* EBs were beating, which was significantly lower than *Nkx2.5$^{+/+}$* cells (Figure 2.3 A). Cardiomyocytes were detected on day 9 by immunofluorescence with antibodies against myosin heavy chain (MHC) (Figure 2.3 B). To determine the percentage of cells that have differentiated into cardiomyocytes, we counted the MHC^{+} cells at day 9 of differentiation. Our result showed that while 30% of the *Nkx2.5$^{+/+}$* cell population was MHC^{+} at day 9 of differentiation, only 15% of the *Nkx2.5$^{R141C/+}$* cells became MHC^{+} (Figure 2.3 C).

This finding is consistent with the previous RT- q PCR results (Figure 2.2 B-C) that showed the down-regulation of core genes required for cardiomyocyte differentiation, resulting in decreased cardiac muscle formation. Together, these results indicate that the presence of the Nkx2.5 R141C mutation reduces the ability of mESCs to undergo cardiomyogenesis.

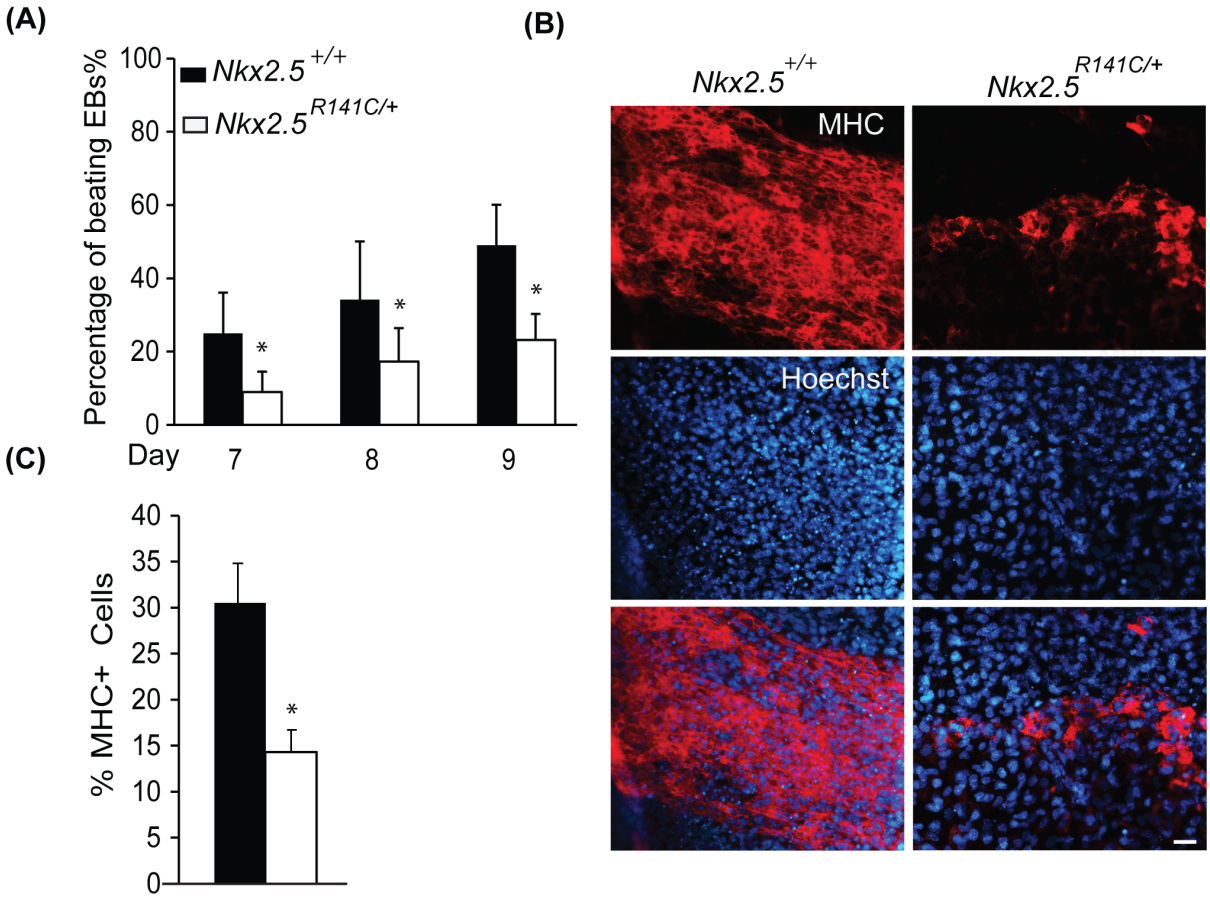


Figure 2.3 *Nkx2.5*$^{R141C/+}$ cells have reduced cardiac muscle formation compared to *Nkx2.5*$^{+/+}$ cells. (A) Quantification of contracting Embryonic bodies (EBs) derived from *Nkx2.5*$^{+/+}$ or *Nkx2.5*$^{R141C/+}$ cells. EBs were transferred to gelatin-coated dish on day 5 and cultured until day 9. The number of contracting EBs (n > 30) was counted per experiment. **(B)** Day 9 differentiated *Nkx2.5*$^{+/+}$ and *Nkx2.5*$^{R141C/+}$ cells were fixed and reacted with MHC-specific antibodies. Nuclei were stained with Hoechst dye. Scale bar = 20 µm **(C)** MHC-positive cardiomyocytes from (B) were counted in 5 random fields per experiment and expressed as percent of the total number of nuclei, Error bars represent ±SEM, (n = 5, *p<0.05).

2.4.4 Defective nuclear accumulation of Nkx2.5 protein in *Nkx2.5*$^{R141C/+}$ cells during cardiomyogenesis and in HL1 cardiomyocyte cells

To determine the protein level of Nkx2.5 protein in *Nkx2.5*$^{R141C/+}$ and *Nkx2.5*$^{+/+}$ cells during differentiation, we extracted whole cell lysates on days 6 and 9 of differentiation. Immunoblot results showed an increase of Nkx2.5 protein level at these two-time points compared to undifferentiated cultures at day 0 (Figure 2.4 A), which is consistent with the *Nkx2.5* mRNA levels as shown by RT-q PCR (Figure 2.2B). However, on day 9 of differentiation, we noticed a slight but not significant decrease of Nkx2.5 protein level in *Nkx2.5*$^{R141C/+}$ compared to *Nkx2.5*$^{+/+}$ cells. Since the Nkx2.5 R141C substitution is within the NLS of Nkx2.5, we assessed the subcellular distribution of Nkx2.5 protein between the nucleus and cytoplasm. The immunoblot result showed that *Nkx2.5*$^{+/+}$ cells have higher

nuclear Nkx2.5 protein compared to cytoplasmic expression. In contrast, we found that Nkx2.5 protein is distributed equally between the nucleus and cytoplasm in *Nkx2.5*$^{R141C/+}$ cells (Figure 2.4 B). To confirm this result, we performed immunofluorescence detection of Nkx2.5 and sarcomeric α-actinin to examine the subcellular distribution of Nkx2.5 protein and its ability to induce the formation of cardiomyocytes. The results showed that Nkx2.5 is mostly localized in the nucleus in *Nkx2.5*$^{+/+}$ cells. However, in *Nkx2.5*$^{R141C/+}$ cells, we noticed a subset of cells that express Nkx2.5 in both the nucleus and cytoplasm (Figure 2. 4 C). This result is in line with the subcellular fractionation results (Figure 2.4 B). Moreover, quantification of total Nkx2.5 and the α-actinin positive cells showed that almost all Nkx2.5^{+} cells express α-actinin protein in *Nkx2.5*$^{+/+}$ cells, which is indicative of cardiomyocyte differentiation. In contrast, only 50% of Nkx2.5^{+} cells co-express α-actinin in *Nkx2.5*$^{R141C/+}$ cells (Figure 2.4 D-E). These results suggest that not all Nkx2.5^{+} cells have the capacity to form cardiomyocytes in *Nkx2.5*$^{R141C/+}$ cells and this may be due to the lower nuclear Nkx2.5 level.

Since the R141C mutation is located within the NLS of Nkx2.5 protein and our previous result showed that the R141C protein is localized in the nuclear and cytoplasm compartments in *Nkx2.5*$^{R141C/+}$ during cardiomyogenesis, we further wanted to investigate whether the mutant protein could be imported to the nucleus in a cardiomyocyte cells. Therefore, we used Leptomycin B (an inhibitor of nuclear export) in HL1 cell line that maintains morphological and biochemical properties of cardiomyocytes (22, 33). In order to do that, HL1 cells were transiently transfected with WT or mutant expression plasmid for 24h. Then, cells

were treated with Leptomycin B (+LMB) or vehicle (-LMB) for 4h, followed by immunofluorescence and quantification of transfected cells that express nuclear or nuclear and cytoplasmic Nkx2.5 protein (Figure 2.4 F-G). Results showed that treatment with LMB, when compared to the vehicle-control, resulted in a significant increase of nuclear accumulation of WT Nkx2.5 protein. In contrast, there was not a significant increase in nuclear accumulations of R141C protein suggesting that it is resistance to leptomycin B treatment (Figure 2.4 F-G). This result suggests that R141C mutation led to impairment of nuclear import of the Nkx2.5 protein in cardiomyocyte cells, and this is in line with what we observed in *Nkx2.5*$^{R141C/+}$ mESCs during cardiomyogenesis.

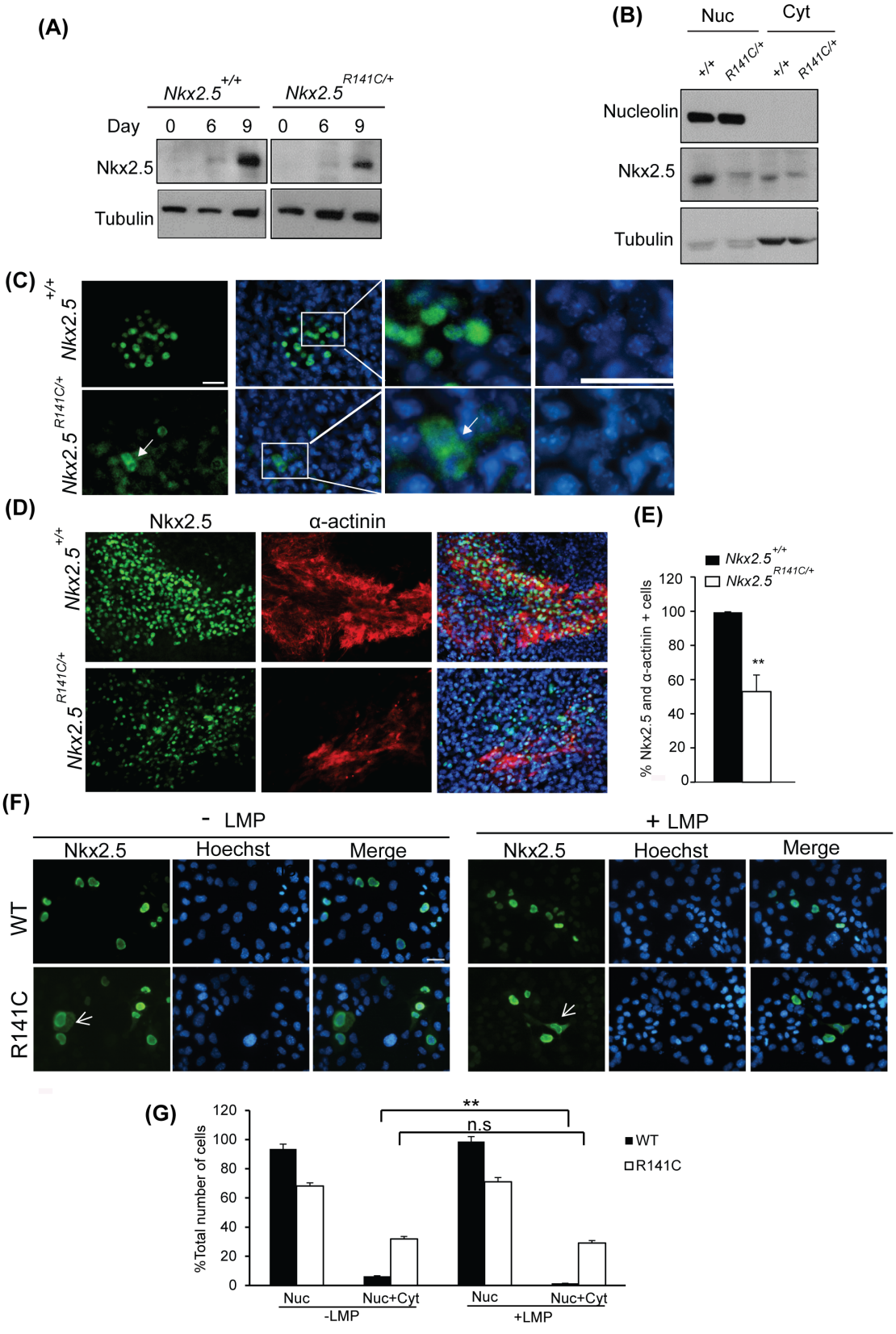

(A)
Nkx2.5+/+
Nkx2.5R141C/+
Day 0 6 9 0 6 9
Nkx2.5
Tubulin
(B)
Nuc
Cyt
+/+
R141C/+
+/+
R141C/+
Nucleolin
Nkx2.5
Tubulin
(C)
+/+ Nkx2.5
R141C/+ Nkx2.5
(D)
Nkx2.5
α-actinin
+/+ Nkx2.5
R141C/+ Nkx2.5
(E)
Nkx2.5+/+
Nkx2.5R141C/+
% Nkx2.5 and α-actinin + cells
120
100
80
60
40
20
0
**
(F)
- LMP
+ LMP
Nkx2.5
Hoechst
Merge
Nkx2.5
Hoechst
Merge
WT
R141C
(G)
%Total number of cells
120
100
80
60
40
20
0
**
n.s
WT
R141C
Nuc
Nuc+Cyt
Nuc
Nuc+Cyt
-LMP
+LMP

Figure 2.4 Nkx2.5 is localized in the nucleus and cytoplasm in *Nkx2.5$^{R141C/+}$* cells during cardiomyogenesis, which is accompanied by a lower number of Nkx2.5^{+} cells expressing α-actinin.

(A) Immunoblot analysis of total protein extracts using Nkx2.5 or α-Tubulin specific antibodies at days 0, 6, and 9. **(B)** Immunoblot of nuclear and cytoplasmic extract using Nkx2.5, Nucleolin and α-Tubulin antibodies at days 9 **(C-D)** Immunofluorescence of *Nkx2.5$^{+/+}$* and *Nkx2.5 $^{R141C/+}$* cells. Cells were fixed on day 9 and co-stained with antibodies specific for Nkx2.5 (green) and α-actinin (red). The arrows indicate the cytoplasmic Nkx2.5 in *Nkx2.5$^{R141C/+}$* cells. The Hoechst dye labels nuclei. Scale bar = 20μm. **(E)** Quantification of Nkx2.5 and α-actinin^{+} cells in *Nkx2.5$^{+/+}$* and *Nkx2.5$^{R141C/+}$* cells (n=4, **p<0.01). **(F)** HL1 cells were transiently transfected with WT Nkx2.5 or R141C expression vector for 24h and then treated with leptomycin B (20ng/ml) for 4h where indicated (+LMB) or with vehicle (-LMB). Cells were fixed and stained with Nkx2.5 antibody (green) and Hoechst dye to detect the nuclei. Scale bar = 20 μm**. (G)** Quantification of cellular distribution of WT Nkx2.5 or R141C mutant protein in **(F)** (Total of 300-400) transfected cells were counted and scored depending on their localization, nuclear (Nuc) or nuclear and cytoplasmic (Nuc+Cyt). The arrows indicate the cytoplasmic Nkx2.5 expression; Error bars represent ±SEM, (n = 3, **p<0.01).

2.4.5 Lower transcriptional activity of Nkx2.5 R141C compared to WT Nkx2.5

Previously it was reported that the *NKX2.5 R142C* mutant has lower transcriptional activity compared to WT NKX2.5 in 10T1/2 fibroblast cells (16). We wanted to further investigate the transcriptional activity of the R141C mutant compared to WT Nkx2.5 using different concentrations of the expression plasmid. P19 embryonal carcinoma cells were transiently transfected with different concentrations of WT Nkx2.5 or R141C mutant expression plasmid along with the reporter construct ANF-luciferase and the SV40-Renilla luciferase plasmid as an internal reference control. Our results showed that while both WT Nkx2.5 and R141C mutant transactivate the ANF promoter in a dosage-dependent manner, the R141C mutant showed significantly lower transactivation of the ANF promoter when compared to the WT Nkx2.5 (Figure 2.5 A). However, when WT Nkx2.5 and R141C were co-transfected at 1:1 ratio we observed a partial but not complete rescue of the transactivation of the ANF promoter (Figure 2.5 B). These results suggest that Nkx2.5 R141C mutant has a significantly reduced transcriptional activity compared with that of the WT Nkx2.5 and does not act as a dominant negative mutation.

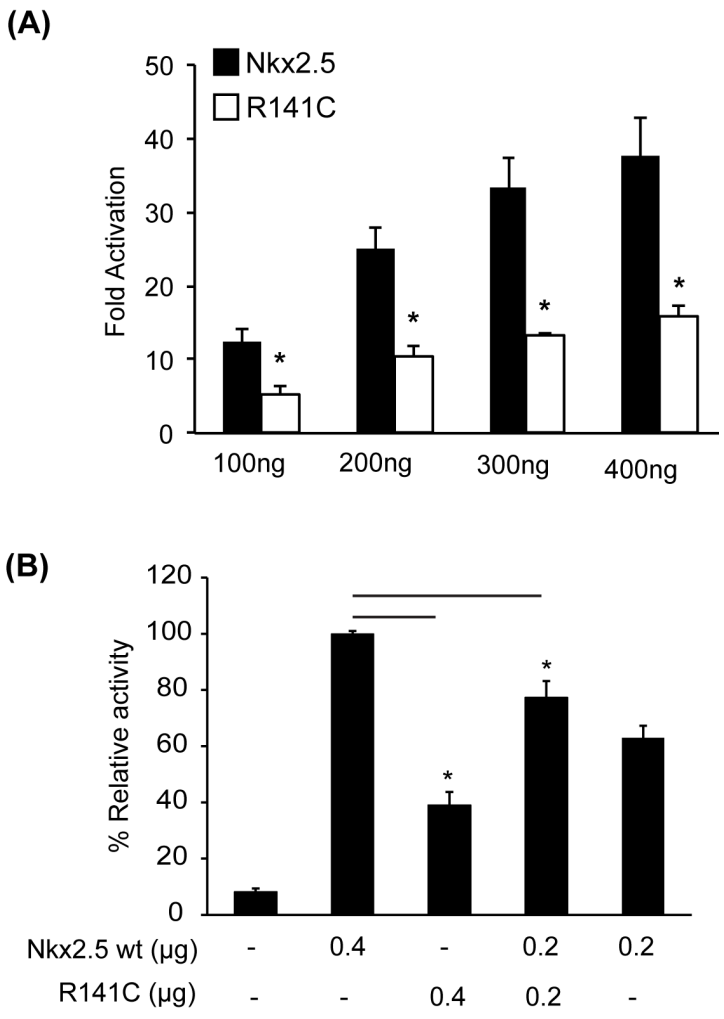


Figure 2.5 Lower transcriptional activity of R141C mutant compared to WT Nkx2.5.

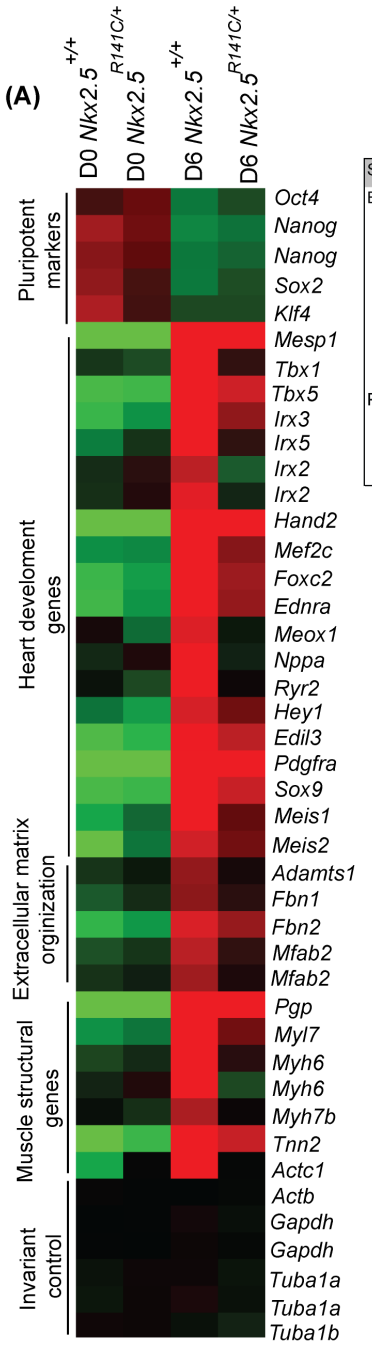
(A) P19 cells were transiently co-transfected with the indicated amounts of WT Nkx2.5 or R141C expression plasmids, along with ANF-luciferase reporter and SV40-Renilla as an internal control. Firefly luciferase activity was normalized to Renilla and reporter activation was quantified relative to the empty vector control. Error bars represent ±SEM, (n=4, *p<0.05). (B) Effect of WT Nkx2.5 and R141C mutant interaction on transcriptional activity. Co-transfection of the WT Nkx2.5 (200ng) and R141C (200ng). Firefly luciferase activity was normalized to Renilla and reporter activation was quantified relative to the empty vector control and data are expressed as a percent of WT Nkx2.5 alone (400ng). Error bars represent ±SEM (n=3, *p<0.05).

2.4.6 Gene expression profiling identifies cardiac genes altered in *Nkx2.5$^{R141C/+}$* cells.

We found that nuclear localization of Nkx2.5 protein, its transcriptional output, and expression of several Nkx2.5 target genes, are all reduced in the presence Nkx2.5 R141C mutant. In order to determine if this reflects a more generalized phenomenon, we used a genome-wide approach to identify altered genes during early stages of cardiomyogenesis. We profiled gene expression by DNA microarray analysis on day 0 and on day 6 at the cardiac progenitor stage when Nkx2.5 is up-regulated in both *Nkx2.5$^{+/+}$* and *Nkx2.5$^{R141C/+}$* cells (34). As invariant controls, *Gapdh* and *alphaTubulin* were unchanged in both mutant and control cell lines (Figure 2.6 A). Moreover, we examined altered genes in *Nkx2.5$^{R141C/+}$* and compared it with control *Nkx2.5$^{+/+}$* cells at day 6 of differentiation. Bioinformatic analysis identified 525 altered genes in *Nkx2.5$^{R141C/+}$* compared to Nkx2.5$^{+/+}$ cells at day 6 of differentiation (with two-fold or greater change in expression in both biological replicates). We used TOPPGENE analysis to identify functional annotation terms significantly enriched among these deregulated genes (35). We found enrichment of biological processes related to heart development, striated muscle contraction, cardiac progenitor differentiation and cardiovascular system development (Figure 2.6 B). Among these altered genes, we found 366 genes that are less induced on day 6 in *Nkx2.5$^{R141C/+}$* compared to *Nkx2.5$^{+/+}$* cells. We confirmed the validity of the microarray data by qRT-PCR on a selection of these genes (Figure 2.6 C). For example, we found several genes important during heart development and

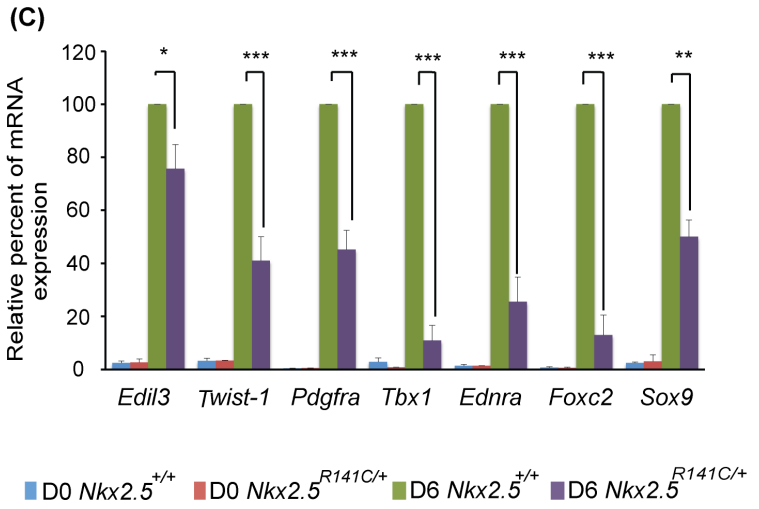
morphogenesis that are up-regulated in the *Nkx2.5*$^{+/+}$ cells show a significant decrease in *Nkx2.5*$^{R141C/+}$ cells, such as *Twist1, Tbx1, Sox9, Edil3, Ednra, Foxc2* and *Pdgfra* (Figure 2.6 C).

We also estimate that our gene expression data can help to identify direct transcriptional targets of *Nkx2.5*. We took advantage of a study that performed ChIP-seq to identify genes bound by *Nkx2.5* in mouse embryos at E11.5 and compared them with our transcriptomic data (36). First, we considered the identity of the 366 genes whose expression is downregulated in *Nkx2.5*$^{R141C/+}$ cells compared to *Nkx2.5*$^{+/+}$ cells (at least 50% decrease in expression, in each of the two biological replicates at day 6), we realized that 41 of them were within 20 kb of binding sites of *Nkx2.5* (ChIP-seq in E11.5 developing hearts). This is a striking enrichment considering that the mouse genome only contains 715 genes with such proximity to *Nkx2.5* binding sites ($p < 1x10^{-8}$, by cumulative hypergeometric distribution test). Second, we found that 117 of the downregulated genes contain in their promoters the hybrid DNA sequence motif bound by *Nkx2.5* and *Meis1* during cardiomyogenesis (Figure 2.6 D), and this also represents a marked enrichment compared to all mouse genome promoters (binomial distribution Z-score of 12.9 using oPOSSUM (37)).



(B)

Source	Category	Targets	FDR B&H	Examples
Biological process	Tissue development	125	6.78E-19	IRX5, MEF2C, PDGFRA, RYR2
	Cardiovascular system development	80	3.12E-17	TBX1, FGF1, HEY1, ZFPM2
	Muscle tissue development	47	2.46E-15	ACTC1, TNNT2, MYH6, MYL2
	Heart development	50	2.19E-13	HAND2, TBX5, FOXC2, FGF1
	Cardiac ventricle development	18	5.40E-08	MEF2C, RYR2, MYOCD, MYH6
	Cardiac chamber morphogenesis	19	4.88E-08	MYBPC3, ADAMTS1, STRA6, TBX3
Pathway	Striated muscle contraction	12	6.97E-07	MYH6., MYL2, MYL3, MYBPC1
	Cardiac progenitor differentiation	10	7.94E-04	TBX5, PDGFRA, FGF2, IGF2
	Extracellular matrix orginaization	20	1.72E-02	FBN1, MFAP2, ADAMTS1, JAM2



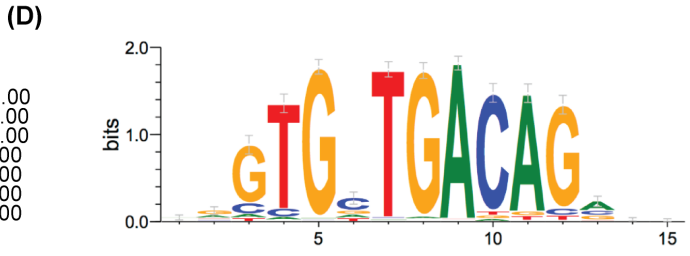


Figure 2.6 Gene expression profiling identifies cardiac genes altered in *Nkx2.5$^{R141C/+}$* cells.

(A) Heatmap of the gene-expression array of *Nkx2.5$^{+/+}$ versus Nkx2.5 $^{R141C/+}$* cells on days 0 and 6 of differentiation. (B) Examples of altered functional gene categories and pathways in *Nkx2.5$^{R141C/+}$* cells. False discovery rate (FDR) was calculated using Benjamini and Hochberg (B&H). (C) Down regulation of several genes important for heart development in *Nkx2.5$^{R141C/+}$* compared to *Nkx2.5$^{+/+}$* cells. qRT-PCR analyses of mRNA expression at days 0 and 6 of both *Nkx2.5$^{+/+}$* and *Nkx2.5$^{R141C/+}$* cells. Data are relative to *Nkx2.5$^{+/+}$* day 0 and represented as percent maximum. Error bars represent ±SEM n=3. *p< 0.05, **p < 0.01,***p< 0.005. (D) The hybrid DNA sequence motif bound by *Nkx2.5* and *Meis1*.

2.5 Discussion

Several studies in humans and mice show that mutations in NKX2.5 are associated with congenital heart defects (8, 14), but the molecular mechanisms underlying the cause of congenital heart defects by these mutations are still poorly understood. In the present study, we discovered that several genes implicated in cardiac development and function are misregulated in *Nkx2.5$^{R141C/+}$* cells compared to *Nkx2.5$^{+/+}$* cells, during the early stages of *in vitro* cardiomyogenesis.

We found that the expression patterns of the mesodermal marker *Brachyury* (*T*) and *Mesp1* in the *Nkx2.5$^{R141C/+}$* were similar to that of the *Nkx2.5$^{+/+}$* cells, suggesting that the Nkx2.5 R141C heterozygous mutant does not interfere

with mesoderm induction. However, at the cardiac progenitor stage, a significant decrease in the mRNA level of *Tbx5* and *Mef2c* was observed. Several studies have demonstrated that *Nkx2.5* regulates target genes that are required for cardiomyocyte differentiation and the conduction system through its interaction with the core transcription factors *Gata-4, Tbx5,* and *Mef2c* (12, 38, 39). Moreover, we showed that Nkx2.5 R141C had reduced transcriptional activity on the ANF promoter compared to WT Nkx2.5 consistent with the previous study (16). We also showed that the Nkx2.5 R141C mutant does not act as a dominant negative. Further, based on the previous *in vitro* study (16), NKX2.5 R142C has reduced capacity to interact with GATA-4, TBX5 and NKX2.5 itself (homodimerization). Therefore, the defect that was associated with $Nkx2.5^{R141C/+}$ could be due to reduced the interaction of Nkx2.5 R141C protein with other cardiac transcription factors (16) and lower transcriptional activity, which resulted in lower transcription of well-known *Nkx2.5* targets (Figure 2.2) such as *Actc1*, *Mlc2v*, and *Anf* (6, 10, 12, 40). The down-regulation of these genes was accompanied by a decrease in the number of beating EBs as well as reduced cardiac muscle formation in $Nkx2.5^{R141C/+}$ cells (Figure 2.3). However, we can not rule out the possibility that the percentage of beating EBs will increase and reach equilibrium with WT as was shown previously in EBs of Nkx2.5 null mESCs (5). We also showed the inability of some $Nkx2.5^{+}$ cells to form cardiomyocytes in $Nkx2.5^{R141C/+}$ cells. These results show that $Nkx2.5^{R141C/+}$ cells undergo cardiomyogenesis less efficiently than $Nkx2.5^{+/+}$ cells consistent with the important role of *Nkx2.5* during cardiac differentiation (39, 41, 42).

Translocation of Nkx2.5 to the nucleus is essential for its function during cardiomyogenesis (17, 43). Also, it has been reported that the nuclear localization of Nkx2.5 is essential for its phosphorylation at serine 163 by casein kinase II (CKII), which results in increased DNA binding and transcriptional activity of Nkx2.5 (13). Interestingly, when the last three amino acids of the NLS were mutated, it led to an increase amount of the cytoplasmic Nkx2.5 protein that was not phosphorylated (13). Given that the R141C mutation is located in the NLS, it was not surprising to find that Nkx2.5 R141C protein is increased in the cytoplasm during cardiomyogenesis (Figure 2.4 C). The higher level of cytoplasmic Nkx2.5 protein in the *Nkx2.5*$^{R141C/+}$ cells compared to *Nkx2.5*$^{+/+}$ cells suggests that a fraction of the total pool of Nkx2.5 protein remains cytoplasmic in some cells during cardiac differentiation. The lower dosage of Nkx2.5 protein located in the nucleus in *Nkx2.5*$^{R141C/+}$ cells could explain the lower transcriptional activity of Nkx2.5 R141C that results in alteration of *Nkx2.5* downstream genes during cardiac differentiation. However, it has been shown that Nkx2.5 R141C protein is located in the nucleus in cells transiently overexpressing this mutation in COS7 cells (16). The difference in the localization of the mutant protein in our work and that previous study (16) could be explained by the fact that different cell types were used (COS7 large T antigen-immortalized fibroblasts *versus* embryonic stem cells undergoing cardiac differentiation or HL1 cardiomyocyte). Also, during cardiac differentiation, Nkx2.5 function is modulated by various signaling pathways, such as Wnt signaling which is known to play a role in modulating Nkx2.5 subcellular localization during

cardiac differentiation (17). The different cell contexts might explain the discrepant results. Together, our results suggest that cytoplasmic Nkx2.5 R141C protein does not translocate to the nucleus as efficiently as the WT Nkx2.5, leading to lower dosage of nuclear Nkx2.5. This, in turn, results in reduced transcriptional activation of its downstream genes (Figure 2.7). Also, based on the previous study that showed that R141C protein has lower binding affinity to DNA compared to WT (16). Therefore, we cannot exclude the possibilities that the lower transcriptional activity of Nkx2.5 R141C is due to both weak DNA binding and dysregulation of cellular localization of the mutant protein.

Microarray analysis revealed aberrant expression of some genes in the *Nkx2.5*$^{R141C/+}$ cell line, including potential downstream targets of *Nkx2.5* that are known to be important for heart development and cardiac muscle formation (Figure 2.6). Our analysis also uncovered new putative transcriptional targets of *Nkx2.5* with potential roles in cardiomyogenesis. Also, given that the *NKX2.5 R142C* heterozygous mutation caused atrial septal defect in humans, we searched our gene expression profiling data for genes that are known to play a role in septal formation during heart development (15). Septal defects are the most common phenotype in patients with heterozygous mutations in the *NKX2.5* gene, which occur as a result of abnormal endocardial cushion (EC) formation (44, 45). Also, null *Nkx2.5* mice do not form an EC, suggesting the need for proper expression of *Nkx2.5* to regulate genes that are important during EC formation (5, 6). Interestingly, our search for genes known to be important for EC formation identified several downregulated genes in *Nkx2.5*$^{R141C/+}$ cells such as

Edil3, Ednra, Foxc2, Twist1 and Sox9 compared to *Nkx2.5*$^{+/+}$ cells (46–48).

NKX2.5 and *MEIS1*, among many genes, are linked to defects in the cardiac conduction system in human, as shown by genome wide association study (49). Interestingly, patients with *Nkx2.5 R142C* heterozygous mutation have a progressive atrioventricular block which causes impairment of conduction system between the atrial and ventricular of the heart (15). Our results suggest that the defect associated with this mutation could be due to an inability of Nkx2.5 R141C mutant protein to reach the nucleus of pre-cardiac mesoderm cells and to bind to target genes in cooperation with Meis1, leading to defects in the formation of the conduction system. A recent study by Bouveret *et al.* has suggested that Nkx2.5 mutant protein can still interact with co-factors and direct it to usual Nkx2.5 targets. At the same time, these co-factors can direct the mutant protein to abnormal “off target genes”, leading to congenital heart disease (50). Therefore, we cannot rule out the possibility that this mutant binds to off-target genes that affect *in vitro* cardiomyogenesis. The identification of *Nkx2.5* target genes whose expression was reduced in *Nkx2.5*$^{R141C/+}$ cell line is consistent with the essential role of *Nkx2.5* in regulating critical genes required for proper heart development and conduction system.

Our study has uncovered, in part, the mechanism underlying Nkx2.5 R141C dysfunction using *in vitro* cardiomyocyte differentiation in mESCs. The impairment of cardiomyocyte differentiation and down regulation of genes required for heart development in *Nkx2.5*$^{R141C/+}$ cells is likely the result of insufficient nuclear localization and lower transcriptional activity of the mutant

protein which led to lowering the dosage that is needed for sufficient transactivation of downstream genes. This results in disruption of the tightly regulated spatiotemporal gene expression pattern during in vitro cardiomyogenesis. Given the complex genetic basis of congenital heart disease caused by *NKX2.5* mutation in general, the identification of candidate genes affecting atrial septal morphogenesis and conduction system defects may advance our understanding of the molecular regulation of congenital heart defects.

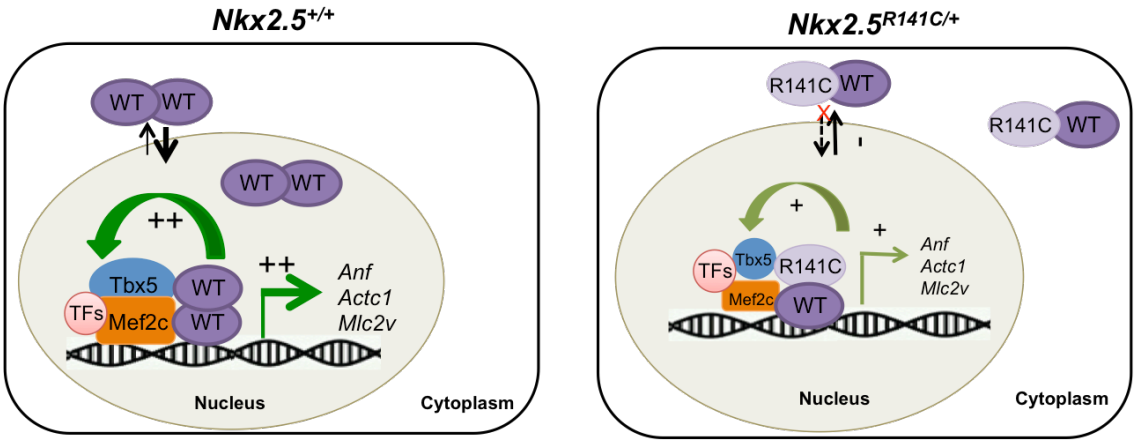


Figure 2.7 Proposed model of the *Nkx2.5*$^{R141C/+}$ cells during cardiomyogenesis compared to *Nkx2.5*$^{+/+}$ cells.

The decreased ability of *Nkx2.5*$^{R141C/+}$ cells to undergo cardiomyogenesis is due to lower transcriptional activity of the R141C mutant on the promoter of downstream genes such as *Anf*, *Mlc2v* and *Actc1*, resulting in decrease cardiac muscle formation. Translocation of Nkx2.5 protein is important for its regulation, in the *Nkx2.5*$^{R141C/+}$ cells, the R141C protein is not properly translocated to the nucleus where it interacts with other transcription factors (TFs) to activate or repress genes important for cardiomyogenesis as the wild type (WT) does.

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CHAPTER 3

Congenital Heart Defect Causing Mutation in *Nkx2.5* Displays *In vivo* Functional Deficit

Abeer F. Zakariyah[1], Rashida F. Rajgara[1], John P. Veinot [6], Ilona S. Skerjanc[1*] and Patrick G. Burgon[3, 4, 5*]

[1] Department of Biochemistry, Microbiology, Immunology, Faculty of Medicine, University of Ottawa, 451, Smyth Road, Ottawa, Ontario, K1H 8M5, Canada.
[2] Ottawa Institute of Systems Biology, Department of Biochemistry, Microbiology and Immunology, Faculty of Medicine, University of Ottawa, 451, Smyth Road, Ottawa, Ontario, K1H 8M5, Canada.
[3] University of Ottawa Center for Neuromuscular Disease.
[4] University of Ottawa Heart Institute, 40 Ruskin Street, Ottawa, Ontario, K1Y 4W7, Canada.
[5] Department of Medicine (Division of Cardiology) & Department of Cellular and Molecular Medicine, Faculty of Medicine, University of Ottawa, Ottawa, Ontario, K1H 8M5, Canada.
[6]Department of Pathology and Laboratory Medicine, Ottawa Hospital

* To whom correspondence should be addressed: iskerjan@uottawa.ca; pburgon@ottawaheart.ca

My contribution to this chapter: I performed most of the experiment in this manuscript with the exception of the functional analyses of the adult mice (ECG and Echo) and the gross heart examination. Rajgara R performed the ECG and I analyzed the results. Richard Seymour performed the Echo and Dr. Burgon P analyzed the results. Dr. Veinot J performed the gross examination of the hearts. The targeting construct and generation of *Nkx2.5*$^{R141C/+}$ mice were designed and created by inGenious Targeting Laboratory. Rajgara R performed the mouse handling. I wrote the manuscript with the contribution of Dr.Patrick Burgon.

This manuscript is in preparation for submission

3.1 Abstract

The Nkx2.5 gene codes for a transcription factor that plays a critical role in heart development. In humans, heterozygous mutations in the *NKX2.5* gene result in congenital heart defects (CHDs). However the molecular mechanisms by which these mutations cause the CHDs are still unknown. *NKX2.5 R142C* is a mutation that is found to be associated with atrial septal defect (ASD) and atrioventricular block (AV block) in 13 patients from one family. The *R142C* mutation is located within both the DNA-binding domain and the nuclear localization sequence (NLS) of the NKX2.5 protein. The pathogenesis of CHDs in humans with *R142C* point mutation is not well understood *in vivo.* To examine the functional deficit associated with this mutation*,* we generated and characterized a knock-in mouse that harbors the human mutation *R142C*. We performed histological examination of the embryonic hearts and newborns, as well as performed functional analysis on the adult mice. We also performed RT-q PCR to examine genes altered in these mice. Our results showed that homozygous embryos with *Nkx2.5 R141C* are developmentally arrested around E10.5 with delayed heart morphogenesis and downregulation of Nkx2.5 target genes. Moreover, $Nkx2.5^{R141C/+}$ newborn mice showed variable morphogenetic cardiac defects and downregulation of ion channel genes that later caused AV block in adult mice. In this study, we replicate a human CHD causing mutation using a mouse model and study the defect associated with this mutation in the homozygous and heterozygous mice during embryogenesis to adulthood. The R141C homozygous mutation is lethal, whereas heterozygosity of this mutation

causes variable cardiac defects and high penetrance AV block and septal defects, which are similar to phenotypes observed in human patients

3.2 Introduction

Congenital heart defects (CHDs) are among the most serious diseases worldwide, affecting approximately 1% of newborns every year (1). Heart development is a complex process that is tightly regulated by transcription factor pathways. Mutations in these transcription factors have been linked to morphological and functional forms of CHDs (2, 3).

Nkx2.5 is a homeodomain cardiac transcription factor that is conserved from flies to humans (4, 5). It is one of earliest cardiac transcription factors expressed in the heart and its expression is maintained to adulthood (6, 7). To date, there are approximately 50 heterozygous mutations identified in the Nkx2.5 gene in patients with CHDs (8). Patients with heterozygous mutations in Nkx2.5 have atrioventricular (AV) conduction blocks and a variety of congenital heart malformations such as atrial septal defect (ASD), ventricular septal defect (VSD), Tetralogy of Fallot (TOF), hypoplastic left heart ventricle, double outlet right ventricle (DORV) arrhythmia and sudden death (9, 10). The pleiotropic cardiac malformation in patients with Nkx2.5 mutation suggests that this transcription factor contributes to several cardiac developmental pathways.

The fundamental role of Nkx2.5 during heart development has been examined using mouse models. Germline deletion of Nkx2.5 causes early embryonic lethality at E10.5 with defects in chamber specification, trabeculation and endocardial cushion formation, suggesting an early requirement for Nkx2.5

function during heart development (11, 12). Nkx2.5 is critical not only during embryogenesis but also after birth and in adulthood. The role of Nkx2.5 has been studied beyond E10.5. For example, mice with tamoxifen inducible Nkx2.5 gene knockout at different stages (E12.5, E19.5, and 2 week old mice) showed morphogenetic cardiac and conduction defects (13–15). Moreover, studies also showed that atrial restricted deletion of Nkx2.5 resulted in cardiac abnormalities including ASD as well as conduction system defects (16), whereas deletion of Nkx2.5 in the ventricle did not cause cardiac defects but resulted in conduction defects including atrioventricular block (17). Nkx2.5 heterozygous knockout mice exhibited different heart abnormalities, such as septal and conduction system defects and the penetrance of ASD was influenced by varied mouse strain(18–21). Recently, a heterozygous mouse model of a human CHD mutation in Nkx2.5 (R52G) resulted in pleotropic cardiac anomalies and conduction defects with higher penetrance, whereas the homozygous mice for this mutation were never born (22, 23).

In this study, we examinethe role of a point mutation in the *NKX2.5* gene *R142C* that has been reported previously in human patients with CHDs (24). The *R142C* mutation was identified in the Nkx2.5 gene in 13 patients from one family who displayed heart abnormalities and conduction defects. The majority of these patients with this autosomal dominant mutation displayed ASD (9/13) and AV block (12/13). The R141C point mutation is located in the N terminus of the Nkx2.5 homeodomain and resulted in substitution of the last amino acid of the NLS (Nuclear Localization Signal) from arginine to cysteine (24). *In vitro*

functional studies showed that this mutation has lower DNA binding, transcriptional activity as well as weak interactions with NKX2.5, GATA4 and TBX5 proteins (25). We also examined the ability of this mutation to modulate cardiomyogenesis by creating knock-in mouse embryonic stem cells (mESC *Nkx2.5*$^{R141C/+}$). Our results showed reduced *in vitro* cardiomyogenesis and alteration of genes important for heart development in Nkx2.5$^{R141C/+}$ (Zakariyah et.al., unpublished data). Therefore, our results along with the previous biochemical study (25) suggested a functional deficit of the R141C mutation *in vitro*.

While Nkx2.5 has been shown to be important for critical processes during heart development, the pathogenesis of CHDs in humans with the *R142C* point mutation is not well understood *in vivo.* In this study, we generated and characterized a knock-in mouse model harboring the human mutation *R142C* in order to examine the functional deficit associated with this mutation *in vivo*. Our results showed that the homozygous mutation of Nkx2.5 R141C resulted in embryonic lethality around E10.5 with delayed heart morphogenesis. Moreover, the *Nkx2.5*$^{R141C/+}$ heterozygous mice displayed variable CHDs. Functional analysis of the *Nkx2.5*$^{R141C/+}$ adult mice showed a high penetrance of AV block. Our study provides a new mouse model harboring the *R142C* mutation in *NKX2.5* gene that is found in human with CHDs.

3.3 Material and methods

3.3.1 Gene targeting and generation of the *Nkx2.5*$^{R141C/+}$ knock-in mouse model

The targeting construct and generation of *Nkx2.5*$^{R141C/+}$ mice (Fig 1A) were designed and created by inGenious Targeting Laboratory (Ronkonkoma, NY). The targeted construct was linearized and electroporated into 129SvES cells. The targeted clones were identified by Southern blotting and direct sequencing. Three clones were injected into C57BL/6 blastocysts to generate chimeric mice. Resulting chimeras were mated to 129sv FLP mice to remove the Neomycin cassette and create somatic Neo deleted mice. Primer set FP and RP were used to screen mice for the deletion of the Neo cassette and further genotyping (Fig 1C). Germline transmission was achieved by mating the somatic Neo deleted mice with 129/SvPasCrl mice from (Charles River).

3.3.2 Breeding and collection of mouse embryos

Mice were mated overnight, and females were examined the following morning for the presence of a vaginal plug. The day that the plug observed was considered as E0.5. *Nkx2.5*$^{R141C/+}$ heterozygous mice were mated to generate *Nkx2.5*$^{R141C/R141C}$ homozygous mice. Pregnant females were sacrifice by cervical dislocation at various embryonic stages. Mice handling and all animal experiments were performed following institutional guidelines and approved by the University of Ottawa for Animal Care and Veterinary Services.

3.3.3 PCR Genotyping

Genomic DNA was extracted from the ear tips of weaned mice or from yolk sacs from embryos with the use of KAPA Mouse Genotyping kit. Primers sequences used for genotyping are as follows: forward primer (FP) 5'-GGG GTA CTT TGA GAC ACT GAG AAC TGC -3', and reversed primer (RP) 5'-CTC CCT ACT CTG AGA TAT TTG GTA TTC GGC -3'. EconoTaq Plus Green 2x Master Mix (Lucigen catalog# 30033-1) was used for the Polymerase chain reaction (PCR) reaction. PCR was done under the following cycling conditions: 94 °C 2 min, (94 °C 30s, 60 °C 30s, 72 °C 1m) for 30 cycles.

3.3.4 Histology and immunofluorescence

P1 neonatal hearts and staged mouse embryos at E10.5 and E9.5 were fixed in 10% formalin, processed, embedded in paraffin, sectioned at 4 μm, and stained with hematoxylin/eosin. For immunofluorescence, embryo sections were deparaffinized, rehydrated and boiled for 5 minutes in 6 mM sodium citrate buffer for antigen retrieval. Sections were blocked in (0.1% BSA, 10% donkey serum in 0.1% Triton X-100/PBS) for 1h at room temperature. Antibodies used: primary antibody Nkx2.5 (goat; 1:50, Santa Cruz; N-19) and secondary antibody (Alexa Fluor 488 donkey anti-goat) at a 1:100 dilution. Pictures were visualized using a Leica DMI6000B inverted fluorescent microscope (Leica Microsystems Inc.).

3.3.5 Gross heart examination

The fixed hearts were examined blind with a dissecting microscope. Under the dissecting microscope, the atria and ventricles were identified and external examination provided orientation and identification of abnormalities. The right

atrium was opened first, and the free wall and interatrial septum were examined for the presence of defects. Then the left atrium was opened. The right ventricle was opened allowing for examination of the ventricular septum for defects. The outflow tract was opened. Finally the left atrium and left ventricle were opened, allowing for examination of the mitral valve, left ventricle outflow tract and the interventricular septum.

3.3.6 Quantitative real time PCR (RT- q PCR)

Total RNA from the hearts of P1 neonate or embryos was extracted using Trizol reagent or E.Z.N.A. ® Micro-elute Total RNA Kit I as per manufacturer's instructions (Omega Bio-tek, USA), respectively. The purified RNA (500ng) was reverse transcribed into cDNA using QuantiTect Reverse Transcription Kit (Qiagen) as per manufacturer's instruction. The KAPA SYBR® FAST Universal qRT-PCR Master Mix (KAPA Biosystems) was used as per manufacturer's instructions and performed on the Mastercycler® Realplex qPCR machine (Eppendorf, Canada). Samples were analyzed in duplicate and the average of the two values was used for further analysis. Expression of candidate genes was normalized to *Gapdh.* Fold change expression values were determined using the delta delta Ct method as described (26) and then normalized to wild type. The error bars represent ± standard error of mean (SEM) of at least three independent biological experiments.

3.3.7 Western blot

Total protein was extracted from hearts of E9.5 mouse embryos and lysed in RIPA buffer (50mM Tris-HCl, pH 8, 150mM NaCl, 1% NP-40, 0.5% Na-deoxycholate, 0.1% sodium dodecyl sulfate) containing phosphatase and protease inhibitor. Antibodies used: primary antibodies against Nkx2.5 (goat; 1:100, Santa Cruz; N-19) and α-Tubulin (1:10.000; DM1A, Sigma-Aldrich). Chemiluminescence was generated using appropriate secondary horseradish peroxidase-conjugated antibodies, anti-mouse (1:5000, Cell Signaling) and anti-goat (1:5000, Santa Cruz), followed by a chemiluminescent reaction using Pierce ECL substrate or SuperSignal™ West Dura Extended Duration Substrate (Fisher Scientific).

3.3.8 Echocardiography and image analysis

Two-dimensional guided M-mode echocardiography was performed in anesthetized (3% isoflurane) 16-20 week old mice using Visual-Sonics VEVO 770 and a 30-MHz linear array transducer. Echocardiography was performed in genotype-blinded fashion.

3.3.9 Electrocardiogram (ECG)

Surface 4-lead ECG devices were implanted subcutaneously in anesthetized (3% isoflurane) 16-20 week old mice. Surface recordings were analyzed using the ECG Auto program (EMKA Technology).

3.4 Results

3.4.1 Generation of the knock-in *Nkx2.5*$^{R141C/+}$ mouse model

The targeting construct (Figure 3.1 A) was used to generate knock-in mice harboring the *Nkx2.5 R142C* mutation. Briefly, the targeting vector contains a mouse genomic fragment for Nkx2.5 from the 129sv strain. The Neomycin cassette flanked by loxP and FRT sites was inserted into the intergenic region, a Flag sequence at the ATG of exon 1 and introduction of (CGC to TGC) non-synonymous substitution at codon 141 (located in exon 2). Positively targeted ES clones were identified by Southern blot and direct sequencing followed by injection into blastocyst to generate chimeras (Figure 3.1 B). Chimeras were the mated to 129sv FLP mice to excise the Neomycin cassette. Germline transmission was achieved by mating to 129/SvPasCrl background and confirmed by PCR and direct sequencing. Primer set FP and RP were used to screen mice for the deletion of the Neomycin cassette and further genotyping (Figure 3.1 C).

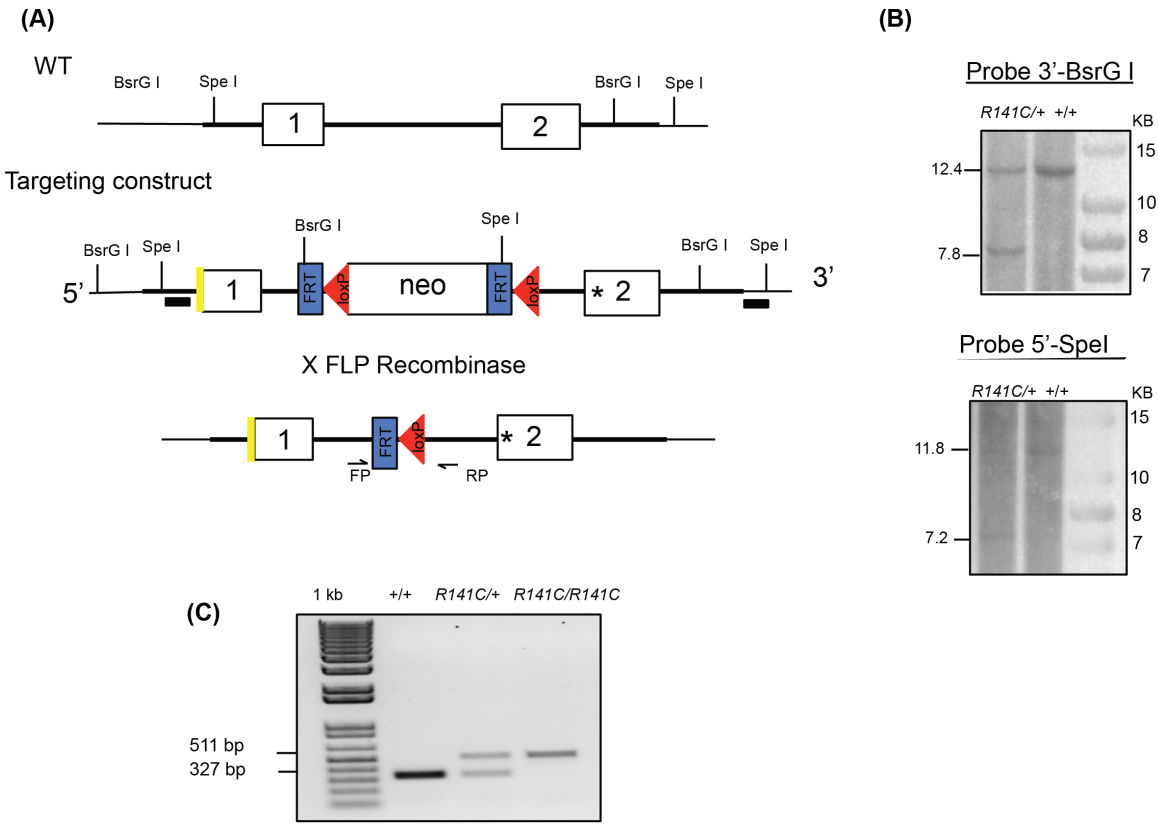


Figure 3.1 Targeting strategy to generate *Nkx2.5*$^{R141C/+}$ knock-in mouse model.

(A) A Structure of WT *Nkx2.5* locus and the targeting construct electroporated in R1 mESCs. Blue (FRT), red (lox), yellow (flag), asterisk (R141C). **(B)** Southern blotting was performed to identify correctly recombined R141C/+ clones. Unique probes from 3' or 5' used and restriction enzyme sites (Spe I) or (BsrG I) were used to digest the DNA respectively. **(C)** Chimeras were mated to 129sv FLP mice to remove the Neo cassette and create Somatic Neo Deleted mice. Primer set FP and RP was used to screen mice for the deletion of the Neo cassette and genotyping. The PCR product for the wild type (WT) is 327 bp. After Neo deletion, one set of LoxP-FRT sites remain (184 bp). A second band with a size of 511 bp indicates Neo deletion in *Nkx2.5*$^{R141C/+}$.

3.4.2 Early embryonic lethality and growth retardation of *Nkx2.5*$^{R141C/R141C}$ homozygous embryos

The *Nkx2.5*$^{R141C/+}$ heterozygote mice appeared grossly normal. In order to examine the *in vivo* functional role of the Nkx2.5 R141C mutation, we intercrossed *Nkx2.5*$^{R141C/+}$ mice to generate *Nkx2.5*$^{R141C/R141C}$ homozygous mice. Interestingly, genotyping results of weaned mice from *Nkx2.5*$^{R141C/+}$ intercrosses failed to show the expected Mendelian ratio and there was an absence of *Nkx2.5*$^{R141C/R141C}$ mice, indicating that the homozygous knock-in allele is embryonically lethal.

In order to screen for the time of embryonic lethality, we set up various timed matings and collected the embryos ranging from E8.5 to E15.5. Genotyping results (Table 3.1) showed that the expected Mendelian ratio of *Nkx2.5*$^{R141C/R141C}$ was observed at E8.5 and E9.5 ($p=0.8$, and $p=0.3$ respectively). However the last survival of the *Nkx2.5*$^{R141C/R141C}$ embryos were observed at E11.5 with $p=0.003$, suggesting that these embryos died in utero and could not survive beyond E9.5-E10.5.

Gross examination and analysis of litters revealed that *Nkx2.5*$^{R141C/R141C}$ mutant embryos were indistinguishable from wild-type (WT) littermates at E8.5 and E9.5 (Figure 3.2 A. a, b and c). However, by E10.5, all *Nkx2.5*$^{R141C/R141C}$ embryos displayed pericardial effusion and appeared to be significantly smaller in body size compared to WT and *Nkx2.5*$^{R141C/+}$ littermates (Figure 3.2 B. a, b and c), which is an indication of growth retardation. Histological examination of WT and homozygous embryos was performed to further investigate the

morphological defects in *Nkx2.5*$^{R141C/R141C}$ embryos. Sagittal sections of embryos at E9.5 (Figure 3.2 A. g, h, i) and E10.5 (Figure 3.2 B. g, h, i) showed that the hearts of all *Nkx2.5*$^{R141C/R141C}$ mice displayed a thinner myocardium wall in the atria and ventricle as well as a decrease or absence of trabeculation in the ventricle, compared to their littermates. Moreover, the hearts of the homozygous embryos at E9.5 and E10.5 showed an absence of endocardial cushion formation (EC) and an absence of epithelial-to-mesenchymal transformation (EMT) of the endocardium in the atrioventricular canal (AVC), which was already evident in the control hearts the WT and heterozygous mice. Theses results suggest that heart development is delayed at E9.5 in the mutant embryo.

Age	# Genotyped	WT	*Nkx2.5*$^{R141C/+}$	*Nkx2.5*$^{R141C/R141C}$	P value
P20	108	42 (39%)	66 (61%)	0	P=0.0001
P1	54	21 (39%)	33 (61%)	0	P=0.0001
E12.5-E15.5	28	9 (32%)	19 (68%)	0	P=0.0001
E11.5	24	11 (46%)	12 (50%)	1 (4%)	P=0.0001
E10.5	34	9 (26%)	21 (62%)	4 (12%)	P=0.007
E9.5	86	23 (27%)	41 (48%)	22 (26%)	P=0.8
E8.5	22	6 (27%)	11 (50%)	5 (23%)	P=0.8

Table 3.1 Genotyping results obtained from intercrossing *Nkx2.5*$^{R141C/+}$ mice

Embryonic lethality of *Nkx2.5*$^{R141C/R141C}$ is shown by the reduced frequencies of *Nkx2.5*$^{R141C/R141C}$ embryos at various developmental stages.

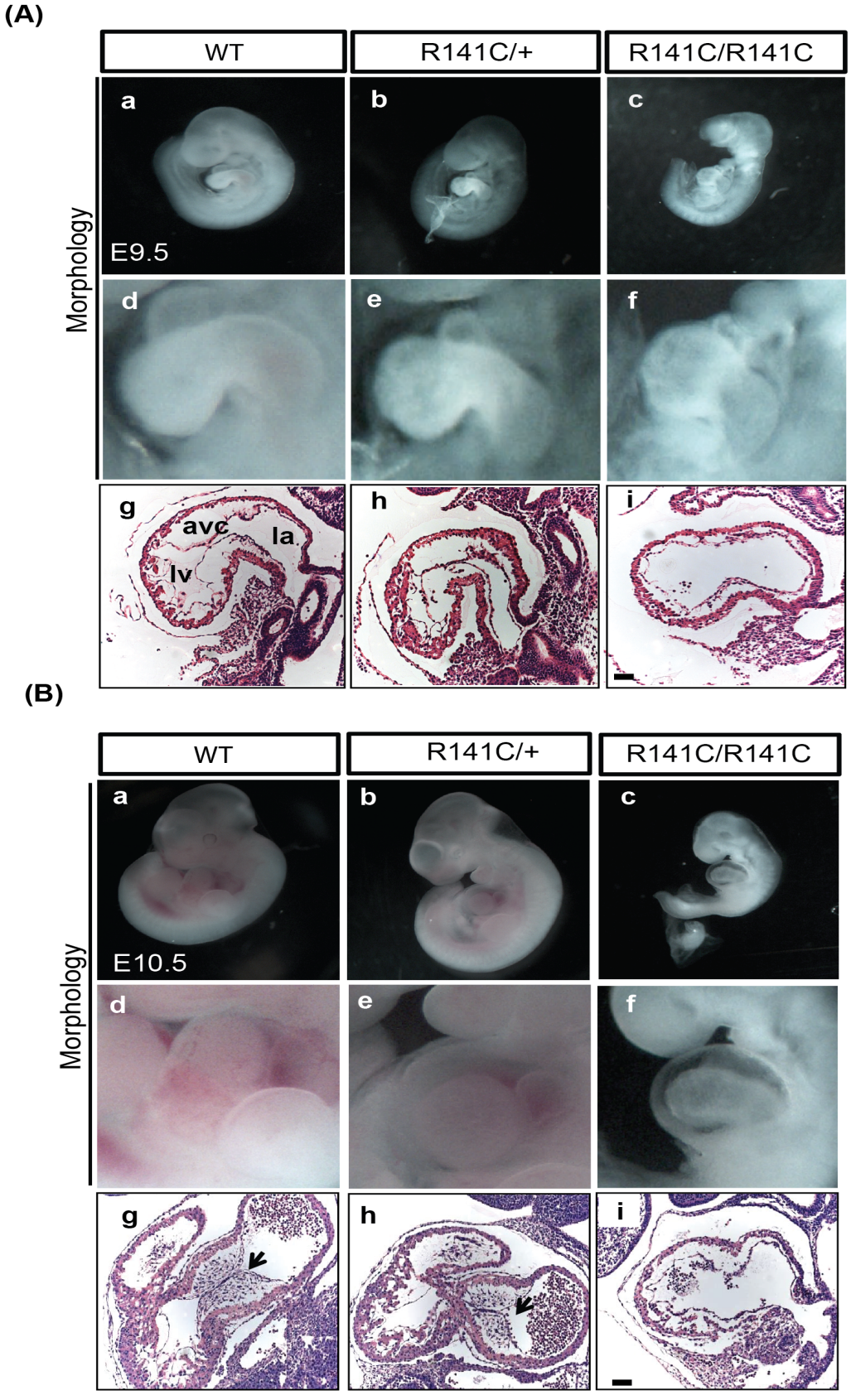

(A)
WT
R141C/+
R141C/R141C
Morphology
a
b
c
E9.5
d
e
f
g
avc
la
lv
h
i
(B)
WT
R141C/+
R141C/R141C
Morphology
a
b
c
E10.5
d
e
f
g
h
i

Figure 3.2 Morphological and histological analysis of *Nkx2.5*$^{R141C/R141C}$ mice displayed growth retardation.

Lateral view of E9.5 (A) and E10.5 (B) whole embryos (a, b and c), and hearts (d, e and f). Hematoxylin and Eosin staining of E9.5 and E10.5 embryos (g, h, and i). Note WT and *Nkx2.5*$^{R141C/+}$ hearts show normal avc at E9.5 (g, h) and normal endocardial cushion E10.5 (g, h) (arrow) but it is absent in *Nkx2.5*$^{R141C/R141C}$ (i). Scale bar = 50 μm (n=3). avc atrioventricular canal, la left atrium, lv left ventricle.

3.4.3 Nkx2.5 R141C protein is expressed in the heart of *Nkx2.5*$^{R141C/R141C}$ embryos.

It is known that Nkx2.5 protein is expressed in the myocardial wall of the heart at E9.5 but is not expressed in the endocardium (6). We examined the expression and localization of Nkx2.5 R141C mutant protein in *Nkx2.5*$^{R141C/R141C}$ hearts by immunofluorescence. Results showed that Nkx2.5 R141C protein was expressed in the myocardium wall of *Nkx2.5*$^{R141C/R141C}$ hearts, as their WT littermates and *Nkx2.5*$^{R141C/+}$ hearts (Figure 3.3 A). We also found that R141C protein was mostly expressed in the nucleus. Moreover, in order to examine the overall Nkx2.5 R141C protein level in the hearts of the E9.5 embryos, we extracted protein from the hearts of WT, heterozygous and homozygous E9.5 embryos. Western blot results showed that total Nkx2.5 protein level in *Nkx2.5*$^{R141C/R141C}$ hearts was expressed at a comparable level as their WT and heterozygous littermates (Figure 3.3 B), suggesting that the R141C mutant mRNA was not undergoing decay and was translated into protein. Although the

hearts of the *Nkx2.5*$^{R141C/R141C}$ embryos looked smaller in size and the myocardium layer was thinner, the Nkx2.5 mutant protein was still expressed similar to WT and heterozygous Nkx2.5 levels.

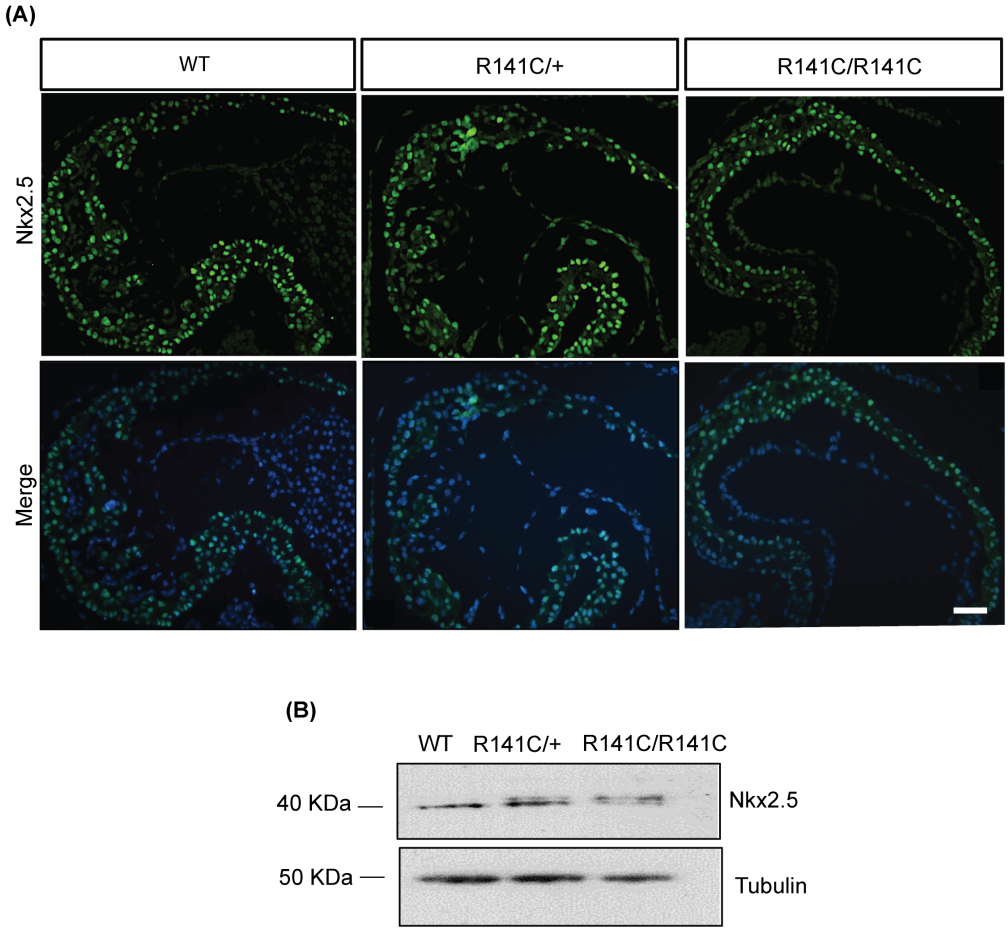


Figure 3.3 Nkx2.5 expression and localization in E9.5 hearts.

(A) Paraffin embedded embryo sections were stained with Nkx2.5 antibody (green) and Dapi to visualize the nucleus. Scale bar=20 μm. avc atrioventricular canal, la left atrium, lv left ventricle. (B) Immunoblot analysis of whole heart lysate from heart of E9.5 WT, Nkx2.5$^{R141C/+}$ and Nkx2.5$^{R141C/R141C}$ using Nkx2.5 or α-Tubulin specific antibodies

3.4.4 Downregulation of genes important for heart development in *Nkx2.5*$^{R141C/R141C}$ embryos

We further examined the mRNA levels of Nkx2.5 and other cardiac transcription factors *Gata4*, *Tbx5 and Mef2c*. We found that all three cardiac transcription factors were significantly downregulated in the heart of *Nkx2.5*$^{R141C/R141C}$ embryos as compared to WT littermates (Figure 3.4 A). The expression of these transcription factors was unchanged in the heart of heterozygous embryos compared to those of WT littermates. Interestingly, we found that the *Nkx2.5* mRNA level is increased in the heart of both homozygous and heterozygous embryos as compared to WT littermates.

Nkx2.5 activates genes important for proper heart formation. R141C has lower transcriptional activity *in vitro* when compared with WT (25). In order to examine the ability of R141C to activate Nkx2.5 target genes *in vivo*, we analyzed the mRNA level of *ANF*, *Mlc2v*, *Actc1* and *Cx40* by q RT-PCR. The expression levels of all four genes were significantly decreased in the hearts of the homozygous embryos as compared to WT controls (Figure 3.4 B). In contrast, the *Mhc7* level, which is not an *Nkx2.5* target, was unchanged. Interestingly, *ANF* and *Mlc2v* levels were both significantly downregulated in the heart of heterozygous embryos but not levels of *Cx40* or *Actc1*.

An important structure that was absent in the *Nkx2.5*$^{R141C/R141C}$ heart was the EC. Therefore, we wanted to evaluate the expression of genes known to be expressed in the EC, such as *Adamts1, Fbn1*, *Msx1*, *Etsrp71*and *Zfpm2* (27–30) (Figure 3.4 C). As expected, all of these genes were significantly downregulated

in the hearts of the *Nkx2.5*$^{R141C/R141C}$ embryos as compared to WT, confirming the absence of EC observed in the histological analysis of these embryos.

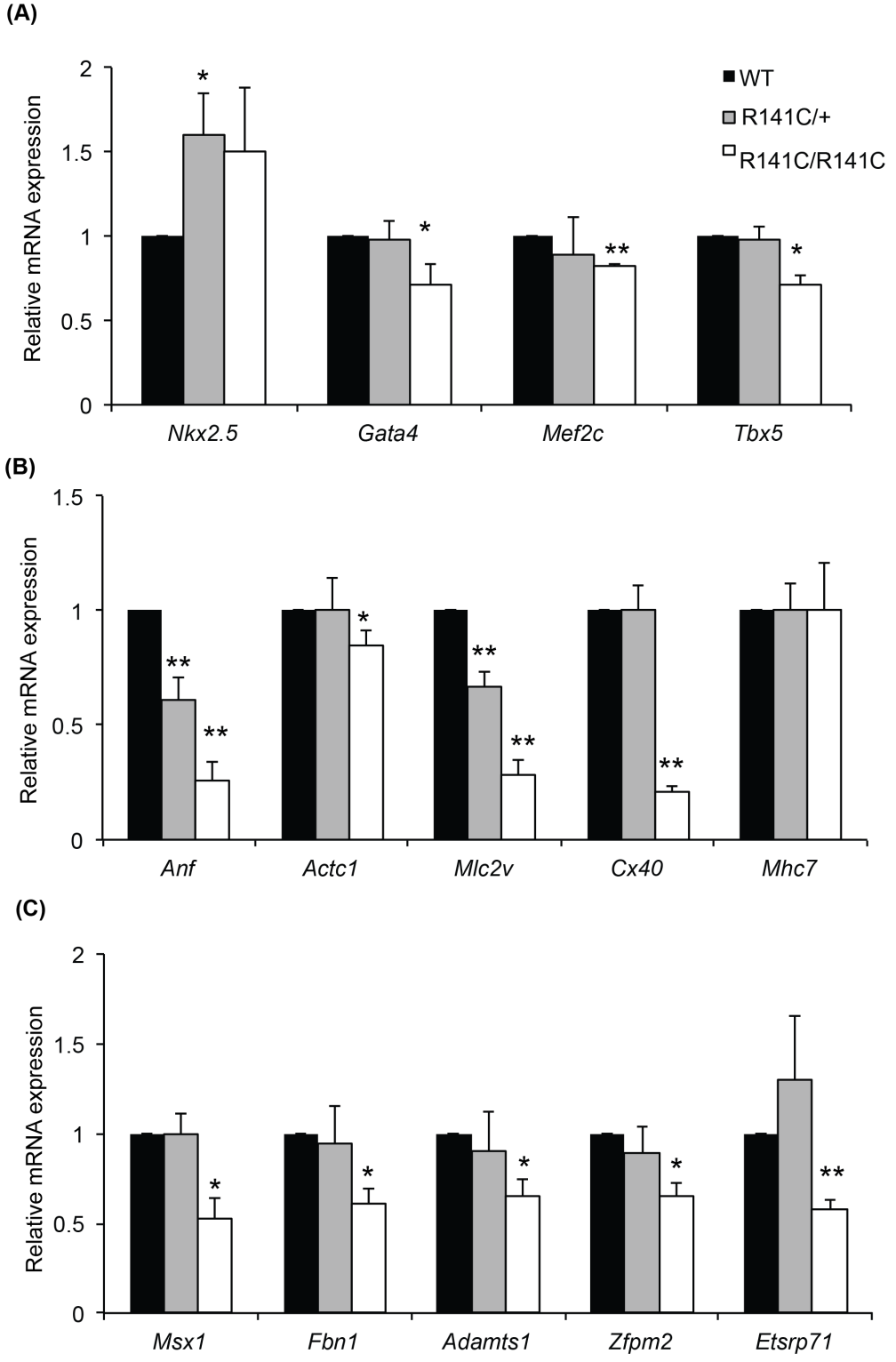


Figure 3.4 Downregulation of genes required for heart development in *Nkx2.5*$^{R141C/R141C}$ embryos.

RNA was extracted from heart of E9.5 embryos. Quantitative RT-PCR analysis of cardiac transcription factors (A), Nkx2.5 target genes (B), and genes important for EMT and EC formation (C). Gapdh was used as a housekeeping gene and all values are compared to the WT. Each experiment is a pool of 3 hearts for each genotype (n=3, *P<0.05, **P<0.001) Error bars represent SEM.

3.4.4 Cardiac malformations in *Nkx2.5*$^{R141C/+}$ mice

It is known that Nkx2.*5* mutations cause a pleiotropic cardiac phenotype in humans (9, 31). The cardiac defects seen in humans with *NKX2.5 R142C* mutations were atrial septal defect 9/13, atrio-ventricular block 12/13, ventricular septal defect 3/13, Tetralogy of Fallot 1/13, and pulmonary valvular stenosis 1/13) (24, 25). Therefore we expected that the *Nkx2.5*$^{R141C/+}$ heterozygous mice would display variable cardiac defects. We found that *Nkx2.5*$^{R141C/+}$ mice were fertile and grossly normal. However, some newborn mice were observed to survive for only a few hours or until postnatal day 1 (P1). Subsequent PCR analyses identified these animals as *Nkx2.5*$^{R141C/+}$ mice. Therefore, we examined the heart morphology of P1 mice to investigate for CHDs.

Newborn mice at P1 were sacrificed and hearts from WT and *Nkx2.5*$^{R141C/+}$ mice were extracted and analyzed by serial histological sections (n=5 WT, n=11 het). Serial histological examination of 11 *Nkx2.5*$^{R141C/+}$ newborn hearts showed that 4/11 (39%) examined hearts displayed ASD (Figure 3.5 A),

while none of the WT P1 hearts examined showed ASD. We also found that some of the *Nkx2.5*$^{R141C/+}$ P1 hearts displayed excessive trabeculation of the left ventricle (Figure 5B). Enlarged right atrium and double outlet right ventricle (DORV) were also observed in several *Nkx2.5*$^{R141C/+}$ mice (Figure 3.5 C-D).

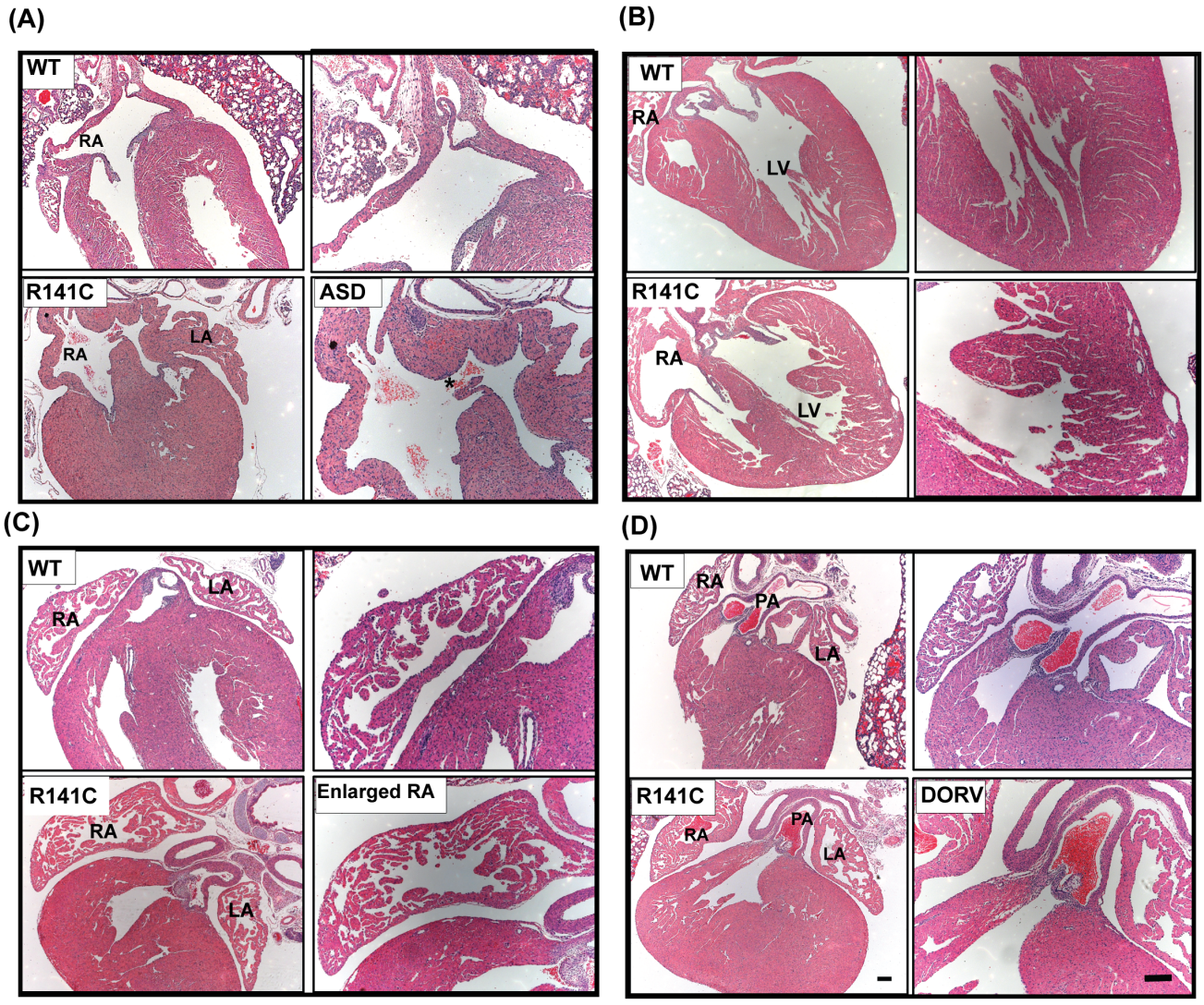


Figure 3.5: Histological analysis of the *Nkx2.5*$^{R141C/+}$ newborn hearts displayed congenital heart defects.

Hematoxylin and Eosin staining of the WT and *Nkx2.5*$^{R141C/+}$ newborn mice. Scale bar = 100 µm. **(A)** Showing an ASD, **(B)** excessive trabeculation of the left ventricle, **(C)** Enlarged RA and **(D)** double outlet right ventricle in *Nkx2.5*$^{R141C/+}$ hearts. (RA) Right atrium, (LA) Left ventricle, (LV) left ventricle, (ASD) atrium septal defect (asterisk), (PA) Pulmonary artery and (DORV) double outlet right atrium.

3.4.6 Gene expression in *Nkx2.5*$^{R141C/+}$ newborn hearts.

We also wanted to test the mRNA levels of known Nkx2.5 downstream genes, cardiac transcription factors and several ion channel genes important for muscle contraction in P1 newborns. To determine if the expression levels of these genes were altered in the *Nkx2.5*$^{R141C/+}$ hearts, we extracted RNA from P1 hearts and analyzed their expression by RT- q PCR. Results showed that there was no difference in the mRNA expression level of cardiac transcription factors *Nkx2.5*, *Gata4*, *Tbx5,* or *Mef2c* (Figure 3.6 A). The mRNA levels of Nkx2.5 downstream genes, *Mlc2v* and *Actc1* in *Nkx2.5*$^{R141C/+}$ hearts were comparable to WT hearts. Moreover, while we noticed a decrease in mRNA level of *Cx40*, we found that the *ANF* mRNA level was significantly downregulated in *Nkx2.5*$^{R141C/+}$ hearts when compared to WT hearts (Figure 3.6 B).

Since Nkx2.5 has an important role in maintaining a normal conduction system, we wanted to further investigate the expression of several ion channel genes known to be important for the cardiac conduction system of which some are Nkx2.5 targets (14, 15). We found a significant reduction in the mRNA level of several important ion channel genes important for electrical polarization of cardiac muscles, such as calcium voltage-gated channel subunit alpha1 C (*Cacna1s*), sodium voltage-gated channel alpha subunit 5 (Scn5a), ATPase sarcoplasmic/endoplasmic reticulum Ca2+ transporting 1 (*Atp2a1*), potassium voltage-gated channel subfamily H member 2 (*kcnh2*), ryanodine receptor 2 (*Ryr2*) and *Id2*. However, the mRNA level of hyperpolarization activated cyclic

nucleotide gated potassium channel 4 (*Hcn4*) in *Nkx2.5*$^{R141C/+}$ hearts was comparable to the WT hearts.

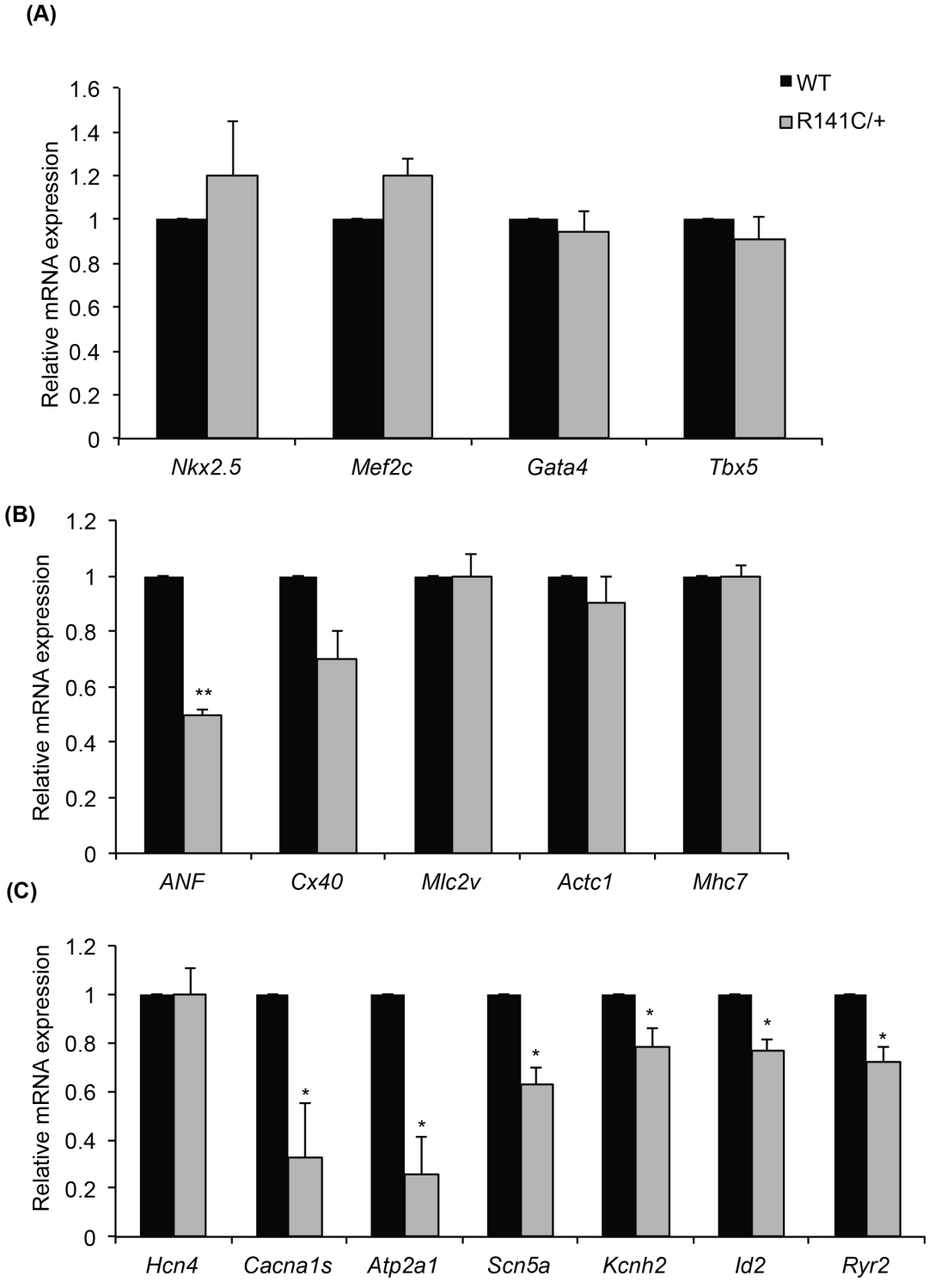
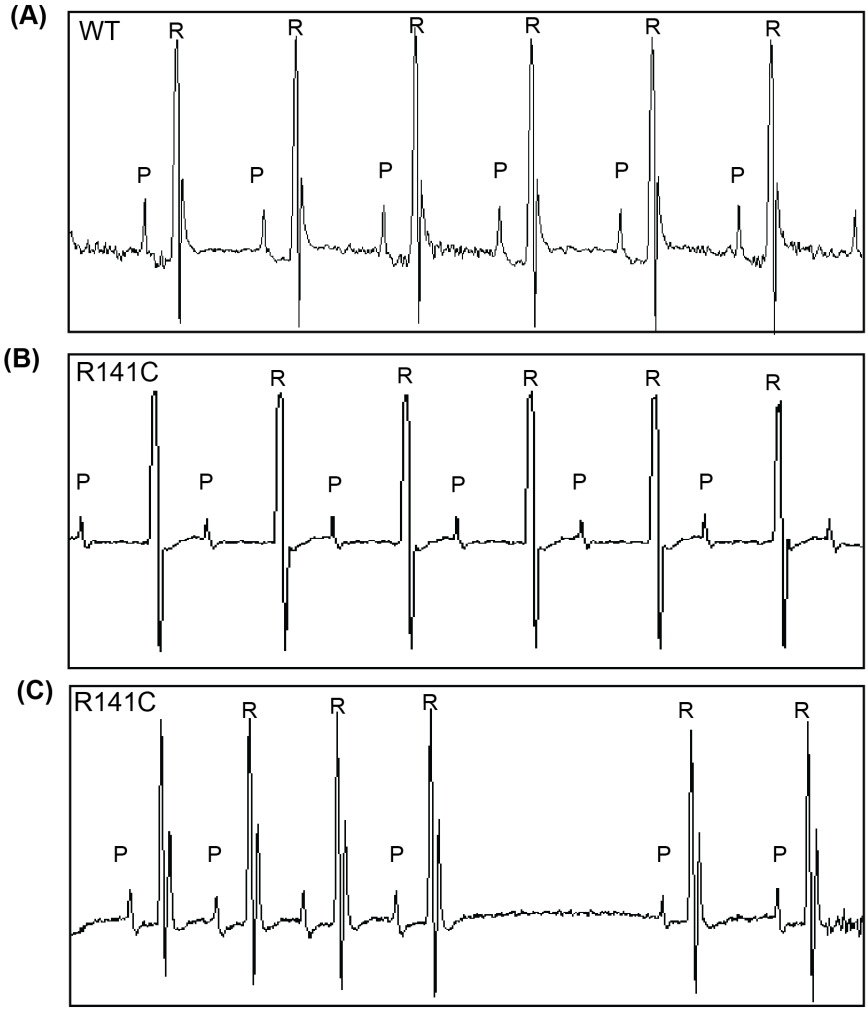


Figure 3.6 Misregulation of genes important for conduction system in *Nkx2.5*$^{R141C/+}$ newborn hearts.

Quantitative RT-PCR analysis of gene expression from newoborns whole heart. **(A)** Cardiac transcription factors, **(B)** Nkx2.5 target genes, **(C)** ion channel genes Gapdh was used as a housekeeping gene and all values are compared to the WT, (n=3, P<0.05), Error bars represent SEM.

3.4.7 Functional analysis of *Nkx2.5*$^{R141C/+}$ adult mice

Although the *Nkx2.5*$^{R141C/+}$ heterozygote mice appeared grossly normal, we examined these mice for cardiac structural and functional abnormalities using electrocardiography and echocardiography. We performed ECGs to examine cardiac contraction abnormalities on adult mice, 16-20 weeks old (WT n=10, *Nkx2.5*$^{R141C/+}$ n=12). Interestingly, we found that most of the *Nkx2.5*$^{R141C/+}$ mice displayed significantly prolonged PR interval (56.2± 4.3 ms), which is an indication of 1st degree AV block versus (37.8±0.67ms) in WT littermates (P=0.001; Student's unpaired t test) (Figure 3.7). We also found 2 of the 12 *Nkx2.5*$^{R141C/+}$ mice tested displayed 2nd degree AV block. Echocardiography analysis of 3-4 month old *Nkx2.5*$^{R141C/+}$ mice revealed no contractile dysfunction or dilation of *Nkx2.5*$^{R141C/+}$ hearts as compared with their control littermates (Table 3.2).



(D)

Surface ECG Recording in *Nkx2.5*$^{R141C/+}$ and WT littermates			
Genotype	**HR**	**PR (ms)**	**QRS (ms)**
Wild type (n=10)	445 ± 11.2	37.8 ± 0.67	12.2 ± 0.63
Nkx2.5R141C/+ (n=12)	435 ± 13.7	56.2 ± 4.3**	12 ± 0.76

Figure 3.7 *Nkx2.5* $^{R141C/+}$ adult mice displayed prolonged PR interval.

(A), (B) and **(C)** ECG recording of (18-20 weeks adult mice) WT (n=10) and *Nkx2.5*$^{R141C/+}$ mice (n=12). **(B)** Showing prolonged PR interval and 1st degree AV block in *Nkx2.5*$^{R141C/+}$ mice. **(C)** 2nd degree AV block, **P value=0.001.

Table 3.2 Echocardiographic measurements of 12-week-old *Nkx2.5*$^{+/+}$ and *Nkx2.5*$^{R141C/+}$ male mice in diastole (d) and systole (s). IVS, inter ventricular septum; LVID, left ventricular internal diameter; FS, fractional shortening; EF, ejection fraction (n=4-5 per genotype, means ± S.D.). Student's t test was used.

	Nkx2.5$^{+/+}$(n=4)	*Nkx2.5*$^{R141C/+}$ (n=5)	P-value
IVSd (mm)	1.11 ± 0.16	1.13 ± 0.17	0.872
LVIDd (mm)	3.48 ± 0.24	3.19 ± 0.51	0.347
LVIDs (mm)	2.39 ± 0.44	2.37 ± 0.47	0.964
%FS	31.8 ± 8.37	26.0 ± 6.05	0.267
%EF	60.1 ± 11.8	52.0 ± 10.0	0.3

3.4.8 *Nkx2.5*$^{R141C/+}$ adult mice display ASD and VSD.

We also examined the hearts of the *Nkx2.5*$^{R141C/+}$ adult mice and their WT littermates for the presence of heart septal defect. Gross examination of the adult hearts showed that the *Nkx2.5*$^{R141C/+}$ heterozygous mice displayed ASD (2/5) in the septum secundum that was not observed in the WT hearts. Also, we found that *Nkx2.5*$^{R141C/+}$ heterozygous mice (3/5) and WT mice (1/5) showed VSD (Table 3.2).

Table 3.3 Representing the frequency of cardiac abnormalities identified in each genotype. ASD, atral septal defect; VSD, ventricular septal defect.

Genotype	VSD	ASD
WT	1/5	0/5
Nkx2.5$^{R141C/+}$	3/5	2/5

3.5 Discussion

The Nkx2.5 R141C mutation has been associated with variable cardiac defects in 13 patients from one family, with the majority displaying ASD and AV block (24). Previous *in vitro* experiments demonstrated that the mutant Nkx2.5 protein had a reduced affinity for DNA binding, with an associated decrease in transcriptional activity and disrupted interaction with critical cardiac transcription factors GATA4, TBX5 and NKX2.5 (25).

Here for the first time, we report the characterization of the R141C mutation in the *Nkx2.5* gene using a knock-in mouse model that replicates a human CHD associated point mutation (24). In this study, we have demonstrated that the R141C mutant, and not some other unknown factor, causes the CHD. We showed that the homozygous mutation Nkx2.5 R141C causes early embryonic lethality and growth retardation by E10.5 and delay in heart development compared to their WT and heterozygous littermates. The ECs were not present in the $Nkx2.5^{R141C/R141C}$ homozygous embryos indicating that EMT is impaired in $Nkx2.5^{R141C/R141C}$ hearts and incomplete ventricular trabeculation. These morphogenetic defects were accompanied by decreased mRNA expression of chamber genes and genes important for EC formation compared to WT hearts. Interestingly, the time of lethality and heart defects seen in our $Nkx2.5^{R141C/R141C}$ homozygous embryos is similar to the defects observed in the $Nkx2.5^{-/-}$ null embryos (11, 12, 19). These result suggest that the *R141C* mutation failed to maintain normal Nkx2.5 function in specification of cardiac

lineage and chamber formation during early cardiac morphogenesis, in which Nkx2.5 is known to have a direct role (17, 30, 32).

Although the Nkx2.5 R141C protein level in the *Nkx2.5*$^{R141C/R141C}$ hearts was comparable to the level found in WT and heterozygous hearts at E9.5, it was not enough to activate Nkx2.5 target genes. Of note, a recent study by Bouveret *et al*. (2015) showed that the Nkx2.5 Y191C mutation still binds to genes that are normally bound by WT Nkx2.5 and has hundreds of DNA sites (off-target) that are not usually bound to WT. Therefore, we cannot exclude the possibility that R141C mutant protein could have other effects and binds to DNA sites that are not normally bound by WT, activating genes that could cause CHDs.

Furthermore, our results showed that during *in vitro* cardiomyogenesis, *Nkx2.5*$^{R141C/+}$ mESCs displayed a partial defect in nuclear localization of Nkx2.5 protein (Chapter 2). However, *in vivo*, R141C protein was mostly localized in the nucleus and we could not detect an obvious defect in nuclear localization when compared to WT. The difference in the localization of R141C protein between the *in vitro* and *in vivo* models could be due to the different time point we examined. It is known that Nkx2.5 is expressed at E7.5 during heart development. Therefore, It is possible that the defect in the nuclear localization of Nkx2.5 protein occurs at earlier stages of heart development *in vivo*, which we have not examined.

We also examined the effect of the heterozygous mutation R141C in the mouse model for the presence of CHDs. Histological examination of the *Nkx2.5* $^{R141C/+}$ mice hearts showed an incidence of ASD (4/11-39%), which was higher

penetrance than that observed in Nkx2.5 heterozygous mice where the incidence of ASD was influenced by mouse strain (18, 20, 21). Our mouse model is a 129sv background, which is known to have a higher incidence of ASD (18, 22). Also, the previous mouse model *Nkx2.5 (R52G)* displayed pleiotropic cardiac anomalies and conduction defects with higher penetrance than the *Nkx2.5*$^{+/-}$ mice (22, 23). The higher penetrance of the CHDs and conduction defects seen in the previously reported *Nkx2.5 (R52G)* and our *Nkx2.5* $^{R141C/+}$ mice suggests that the presence of a point mutation in one allele of Nkx2.5 gene could have a broader effect on Nkx2.5 function than the null allele in the *Nkx2.5*$^{+/-}$ mice.

Abnormal trabeculation in the ventricular wall was also observed in the *Nkx2.5* $^{R141C/+}$ mice. Several reported Nkx2.5 transgenic mice showed hypertrabeculation of the ventricular wall (13, 17). The morphogenic defects observed in *Nkx2.5* $^{R141C/+}$ newborn hearts suggests that an alteration of the Nkx2.5 direct or indirect target genes occurred during early heart development that resulted in various CHDs. For example, the mRNA levels of *Mlc2v and Anf* were downregulated in the hearts of the E9.5 heterozygous mice when compared to WT hearts, which are both known to play an important role in ventricular chamber morphogenesis (33–35). The expression of *Anf* was downregulated during heart development in both mice with Nkx2.5 point mutations, *Nkx2.5*$^{R141C/+}$ and *Nkx2.5 (R52G)*, but was not affected in the *Nkx2.5*$^{+/-}$ heterozygous mice (36). These results also indicate that during heart development, the point mutation in *Nkx2.5* gene affects its downstream targets, which could affect the tightly regulated gene network during heart development leading to various CHD

phenotypes and conduction defects (9, 37). This result could explain the higher penetrance of the cardiac and conduction defects seen in mice with point mutation in *Nkx2.5* gene compared to the *Nkx2.5*$^{+/-}$ heterozygous mice that have a milder phenotype. However, the *Mlc2v* mRNA level in the *Nkx2.5*$^{R141C/+}$ heterozygous hearts was comparable to that in WT newborn mice, suggesting the existence of mechanisms able to compensate for the R141C allele.

Most *Nkx2.5*$^{R141C/+}$ mice survived to adulthood but showed conduction defects. Most of the adult *Nkx2.5*$^{R141C/+}$ heterozygous mice examined developed a prolonged PR interval, which was sufficient to produce a 1st degree AV block. We also found that 2 out of 12 tested mice showed 2nd degree AV block, which was not observed in their age-matched controls. Our results also showed misregulation of ion channel genes required for cardiac contraction, which was in accordance with the conduction abnormalities observed in these mice. These results are consistent with what has been observed in human patients with the *R142C* mutation displaying AV block with high penetrance. Although we did not observe a 3rd degree AV block in the *Nkx2.5*$^{R141C/+}$ mice examined, it is still possible that these mice will develop more progressive AV block as they age. The presence of an AV block suggests defects in the conduction system and in the AV node which have been demonstrated in several Nkx2.5 heterozygous and conditional knockout mice (13–17, 19, 21, 23, 38)

In summary, this study characterized a human disease causing mutation in the *Nkx2.5* gene from embryo stage to adulthood using a knock-in mouse model harboring the disease. This mouse model will be a valuable tool for

understanding the mechanisms causing CHDs and could be used to identify misregulated or off-target genes linked to the *R141C* mutation, which will further improve our understanding of phenotype/genotype correlations or screening for CHDs.

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CHAPTER 4

The Interaction of the Murine Nkx2.5 R141C Mutant with the Myosin Phosphatase Complex

Abeer Zakariyah, Ashraf Al Madhoun[2], Ilona Skerjanc[1,*] and Patrick Burgon[3,4,*]

[1] Department of Biochemistry, Microbiology, Immunology, Faculty of Medicine, University of Ottawa, 451, Smyth Road, Ottawa, Ontario, K1H 8M5, Canada.

[2] Dasman Diabetes Institute. Research Division, 1180 Dasman, Kuwait.

[3] University of Ottawa Heart Institute, 40 Ruskin Street, Ottawa, Ontario, K1Y 4W7, Canada.

[4] Department of Medicine (Division of Cardiology) & Department of Cellular and Molecular Medicine, Faculty of Medicine, University of Ottawa, Ottawa, Ontario, K1H 8M5, Canada.

* To whom correspondence should be addressed: iskerjan@uottawa.ca; pburgon@ottawaheart.ca

My contribution to this Chapter: I performed all the reported experiments in this Chapter and I wrote the manuscript. Dr. Ashraf Al Madhoun helped with the generation of the stable P19 cells.

This manuscript is in preparation for submission.

4.1 Abstract

Nkx2.5 is a transcription factor that is critical to regulate cardiac gene expression during heart development. A previous study in our laboratory has found that myosin phosphatase (MP) complex subunits PP1β and Mypt1 are novel binding partners of Nkx2.5 protein during cardiomyogenesis in stem cells. The interaction of Nkx2.5 with the MP complex resulted in exclusion of Nkx2.5 protein from the nucleus to the perinuclear region and a decrease in Nkx2.5 transcriptional activity. Nkx2.5 protein has a PP1-binding consensus sequence, RVxF, located at the N-terminus of the homeodomain. Notably, the PP1-binding sequence, RVxF, is mutated from arginine to cysteine in the amino acid 142 in human patients who displayed atrial septal defects and atrioventricular block. We hypothesized that the wild type (WT) Nkx2.5 binds to this MP complex through the RVxF motif and that the *R142C* mutation will not bind to it. In the present study we focus on examining the ability of the R141C mutation (the murine homologue of the human *R142C* mutation) to interact with the endogenous MP complex in P19 cells. Our results showed that the R141C protein interacts with the MP complex, but the transcriptional activity of the R141C mutation does not change in the presence of the MP complex. Also, the R141C protein is not translocated to the perinuclear region in the presence of MP complex as the WT Nkx2.5 does. In conclusion, this study shows that the R141C mutant interacts with the MP complex but shows resistance to MP inhibition and localization. Moreover, Interaction of Nkx2.5 with MP is complex and does not fully require the Arginine in the RVxF motif in Nkx2.5 protein.

4.2 Introduction

In humans, heterozygous mutations in *NKX2.5* are associated with congenital heart defects (CHDs), but the molecular mechanisms underlying these defects are still unknown. Previous work in our laboratory identified a novel interaction between the cardiac transcription factor Nkx2.5 and the myosin phosphatase (MP) enzyme complex during cardiomyogenesis in P19 and mouse embryonic stem cells (mESCs) (1). They showed (1) that Nkx2.5 interacts specifically with the phosphorylated form of MP, which resulted in Nkx2.5 protein exclusion from the nucleus to the perinuclear region and a decrease in Nkx2.5 transcriptional activity in P19 cells. It is also known that Wnt3a inhibits terminal cardiomyogenesis and modulates Rho-associated kinase (ROCK) activity (2,3). Treatment of mESCs with Wnt3a at a later stage of cardiac differentiation resulted in nuclear exclusion of Nkx2.5 and its co-localization with pMypt1T853 as well as downregulation of cardiac genes and reduced cardiomyogenesis. In summary, these results suggest that Wnt3a activates ROCK, which phosphorylates Mypt1 at Thr853, resulting in its movement to the nucleus where it interacts with Nkx2.5. Subsequently, the MP/Nkx2.5 complex is exported to the perinuclear region, which is accompanied by reduced cardiomyogenesis (1).

The MP holoenzyme complex is composed of two subunits: the catalytic subunit, protein phosphatase 1β (PP1) and the regulatory targeting subunit 1, myosin phosphatase (Mypt1) (4). The MP complex modulates the balance of phosphorylation of the regulatory light chain of myosin II with the myosin light chain kinase, thereby regulating muscle contraction (5). PP1 regulatory proteins

contain RVxF, a primary PP1-binding motif, which interacts with the targeting subunits (6,7). Mypt1 has a KVKF motif, located near the N-terminus, which allows it to dock onto PP1 to form the MP complex. PP1β also interacts with Mypt1 through another consensus sequence known as MyPhoNE, as well as SILK, located in the N-terminus of Mypt1 (4,7–9). ROCK phosphorylates Mypt1 at Thr853 and results in a redistribution of the MP complex to the perinuclear region and the nucleus (10). In addition, this phosphorylation event inhibits the activity of the PP1β catalytic subunit, due to docking of the phosphorylated Mypt1 residue within the active site of the catalytic subunit (11).

Nkx2.5 contains a conserved PP1 binding sequence, known as RVxF (12) located at the N-terminal region of the homeodomain (HD); amino acid 142-145 and 141-144 in human and mouse, respectively. A heterozygous point mutation in *NKX2.5* gene, the *R142C* mutation, was reported in 13 patients from one family where patients suffered from atrial septal defect and atrioventricular block. The patients with the *R142C* mutation had the conserved arginine “R” in the position 142 of the HD of the *NKX2.5* mutated to cysteine “C” (*R142C*) (13,14).

The aim of this study was to investigate whether the mutant of Nkx2.5 (R141C) would still bind to the MP complex in P19 cells. Of note, we used the term R141C mutation because it is the murine homologue of the human *R142C* mutation. We also examined if the presence of the MP complex would have a similar effect on the R141C mutant transcriptional activity and localization as it did for the WT Nkx2.5. In the present study, our results revealed that the mutant

R141C protein interacts with the endogenous MP complex in P19 cells. However, unlike the WT Nkx2.5, the transcriptional activity of the mutant is not reduced, and no change in cellular localization was observed for the R141C protein. These results suggest that the R141C mutant responds differently than the WT Nkx2.5 protein in the presence of the MP complex. Understanding the molecular function of the R141C may provide us with more insight into how this mutation causes the disease.

4.3 Material and methods

4.3.1 Plasmids

Flag-WT Nkx2.5, Myc-Mypt1 and HA-PP1 plasmids have been previously described (15–17). The R141C mutant was generated using the Quik Change II-XL kit, as per the manufacturer's instructions. We generated the point mutation using the mouse Nkx2.5 coding sequence. Of note, the arginine is located at amino acid 141 of the mouse Nkx2.5 coding sequence; therefore we will name the mutation R141C hereafter. The ANF reporter has been previously described (18), SV40-Renilla was from (Promega).

4.3.2 Cell culture and generation of P19 stable cell lines.

P19 embryonal carcinoma cells were obtained from ATCC, #CRL-1825. Cells were cultured in α-Minimum Essential Media supplemented with 10% fetal bovine serum, 100U/ml streptomycin and 100µg/ml penicillin. For the generation of the stable cell lines, P19 cells were transfected with the DNA constructs Flag-

WT Nkx2.5, Flag-R141C or the Flag-empty vector (EV), along with PGK-puro, and B17 (19, 20) using the *Fugene* 6 transfection kit (Roche Diagnostics Canada, Quebec) as per manufacturer's instructions. Twenty-four hours after transfection, cells were grown under puromycin selection for 7 days. Then individual colonies were analyzed for Nkx2.5 mRNA and protein expression.

4.3.3 Reporter assay

To examine the effects of Mypt1/PP1 on the transcriptional activity of R141C, P19 cells were transiently co-transfected with 200ng Flag-WT Nkx2.5 or Flag-R141C with or without 200ng Myc-Mypt1 and 200ng HA-PP1, along with 100ng of ANF-luciferase reporter and 50ng of SV40-Renilla as an internal control. The total plasmid concentration was adjusted to 1µg with an EV. Transfected cells were harvested after 24h and luciferase assays were conducted as per manufacturer's instructions (Dual-Luciferase® Reporter Assay System, Promega). Samples were analyzed on an LMax II 384 luminometer (Molecular Devices). Firefly luciferase activity was normalized to Renilla and reporter activation was quantified relative to the empty vector control. The protein lysates from this transfection were saved for further immunoblot analysis.

4.3.4 Co-immunopreciptation and immunoblot

Protein lysates were collected from P19 cells overexpressing Flag-WT Nkx2.5, Flag-R141C or Flag-EV and immunoprecipitated using a FLAG® Immunoprecipitation Kit (*SIGMA-ALDRICH, Germany*). The beads were washed and the immunoprecipitated proteins were eluted by boiling in sample buffer.

Input and immunoprecipitated (IP) samples were analyzed by immunoblot using antibodies directed against: Nkx2.5 (goat; 1:100, Santa Cruz; N-19), Mypt1 (Cell Signaling, Danvers, MA) or PP1 (MILLIPORE).

4.3.5 Immunofluorescence

P19 cells were co-transfected with Flag- WT Nkx2.5 or Flag-R141C in the presence or absence of HA-PP1 and myc-Mypt1. After 24h, cells were washed and fixed with acetone and then blocked in blocking solution (0.1% BSA, 10% donkey serum in 0.1% Triton X-100/PBS) for one hour at room temperature. Fixed cells were then incubated with primary antibodies anti-Nkx2.5 (goat; 1:100, Santa Cruz; N-19), or anti-Myc overnight at 4°C. After washes with PBS, the secondary antibodies were applied (1:100 dilution in PBS) for one hour at room temperature (Alexa Fluor 488 donkey anti-goat or Cy3 donkey anti-mouse). Hoechst dye (Sigma-Aldrich, cat.no.B-2883) was used to counterstain nuclei. Cells were then visualized using a Leica DMI6000B inverted fluorescent microscope (Leica Microsystems Inc.).

4.4 Results

4.4.1 The R141C mutant has lower transcription activity than the wild type and is not inhibited by the MP complex

In order to examine the transcriptional activity of the R141C mutant in the presence of the MP complex; P19 cells were transfected with WT Nkx2.5 or R141C in the presence or absence of Mypt1 and PP1, along with ANF-luciferase and SV40-Renilla. The results showed that while both the WT Nkx2.5 and the R141C mutant enhanced the ANF promoter activity; the R141C mutant exhibited a significant lower transactivation of the ANF promoter as compared to the WT Nkx2.5, $*p < 0.05$, in agreement with previous reported results (14) and our result (Figure 2.5 in Chapter 2).

In the presence of MP complex, a 25% reduction in the ANF promoter-luciferase activity was observed with WT Nkx2.5 co-transfections. On the other hand, no changes were detected in ANF promoter-luciferase activity using R141C mutant co-transfected with or without MP complex (Figure 4.1). Thus, although the R141C mutant displays a reduced activation of the ANF reporter gene, it is resistant to the inhibition by MP complex.

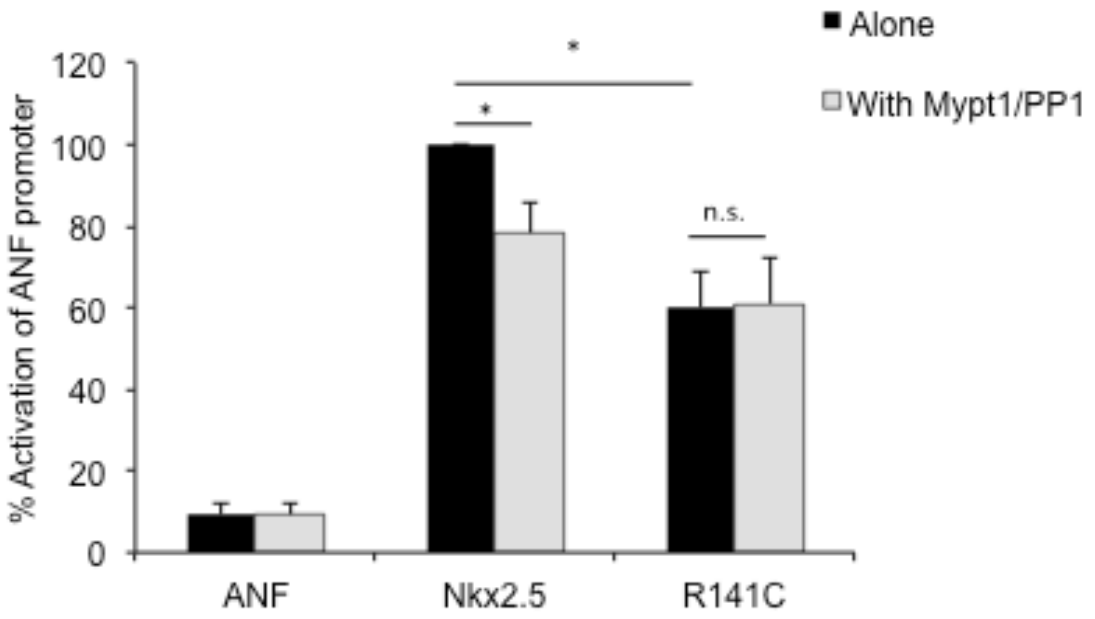


Figure 4.1: The R141C mutant has lower transcriptional activation on the ANF promoter than the wild type Nkx2.5 that is not inhibited by the MP complex.

P19 cells were transiently transfected with Flag-WT Nkx2.5 or Flag-R141C mutant alone or in the presence of Mypt1 and PP1, along with the ANF-luciferase reporter and SV40-Renilla, as an internal control. Firefly luciferase activity was normalized to Renilla and reporter activation was quantified relative to the empty vector control and data were expressed as a percent of Nkx2.5 alone, n.s. not significant, Error bars represent ± SEM, *$p<0.05$, n=4.

4.4.2 The R141C mutant protein is not translocated to the cytoplasm in the presence of the MP complex in P19 cells

To investigate the subcellular localization of the R141C mutant, P19 cells were transiently transfected with Flag-WT Nkx2.5 or Flag-R141C alone or with HA-PP1 and the Myc-Mypt1 construct. We determined the subcellular localization of the WT Nkx2.5 or R141C protein in the co-transfected cells with the MP complex and compared them with cells transfected with WT or R141C alone. As previously reported (1), around 25% of the WT Nkx2.5 protein moved to the perinuclear region in the presence of the MP complex when compared to cells transfected with Nkx2.5 alone, *$p<0.05$. Interestingly, we found that the R141C mutant protein did not move into the perinuclear region in the presence of the MP complex when compared to cells transfected with R141C alone (Figure 4.2 A-B). These data indicate that the R141C mutant is resistant to the MP-induced nuclear exclusion. Notably, the R141C mutation is also located in the last amino acids of the nuclear localization signal (NLS) (amino acid sequence: RRRRKPR), which is believed to affect the subcellular localization of Nkx2.5 protein (21). However, the R141C mutation did not prevent nuclear localization and was predominantly localized in the nuclei of P19 cells as was shown also in a previous study (14).

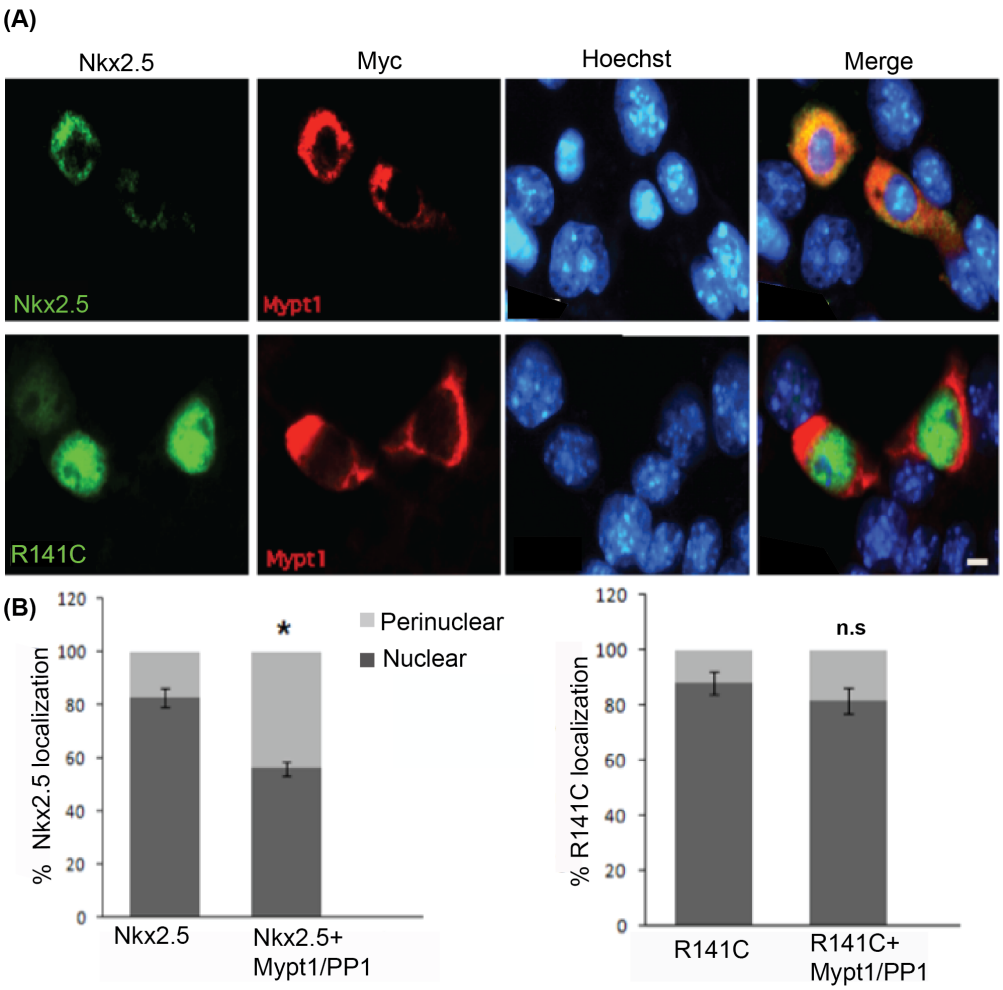


Figure 4.2: R141C mutant is not translocated to the cytoplasm in the presence the MP complex in P19 cells.

P19 cells were transiently co-transfected with Flag-Nkx2.5 or Flag-R141C with or without myc-Mypt1 and HA-PP1, and fixed two days later for analysis of subcellular localization. **(A)** Fixed cells were labeled with antibodies specific to Nkx2.5 (green) and Myc (red) as well as with Hoechst dye (blue) to detect nuclei. Scale bar represents 20μm. **(B)** Subcellular localization of Nkx2.5 WT and R141C was quantified by imaging 5 fields of 4 independent experiments and a total of approximately 150 cells were counted per treatment, n.s not significant, *p<0.05.

4.4.3 The presence of MP reduces WT Nkx.5 but not R141C protein levels

To examine the levels and stability of the Nkx2.5 WT or R141C proteins in the presence of the MP complex; western blot analysis of P19 cells transfected with Nkx2.5 or R141C in the presence or absence of Myc-Mypt1 and HA-PP1 was performed. After 24 hours of transfection, total protein lysates were harvested and analyzed by western blot with an antibody specific to Nkx2.5. We found that the protein levels of the R141C mutant protein did not change in the presence, of the MP complex. In contrast, the WT Nkx2.5 protein levels were significantly reduced by about 40% in the presence of MP complex (Figure 4.3). Thus, Unlike the R141C mutant which showed protein stability in the presence of MP complex; the nuclear exclusion of Nkx2.5/MP protein complex is associated with protein degradation.

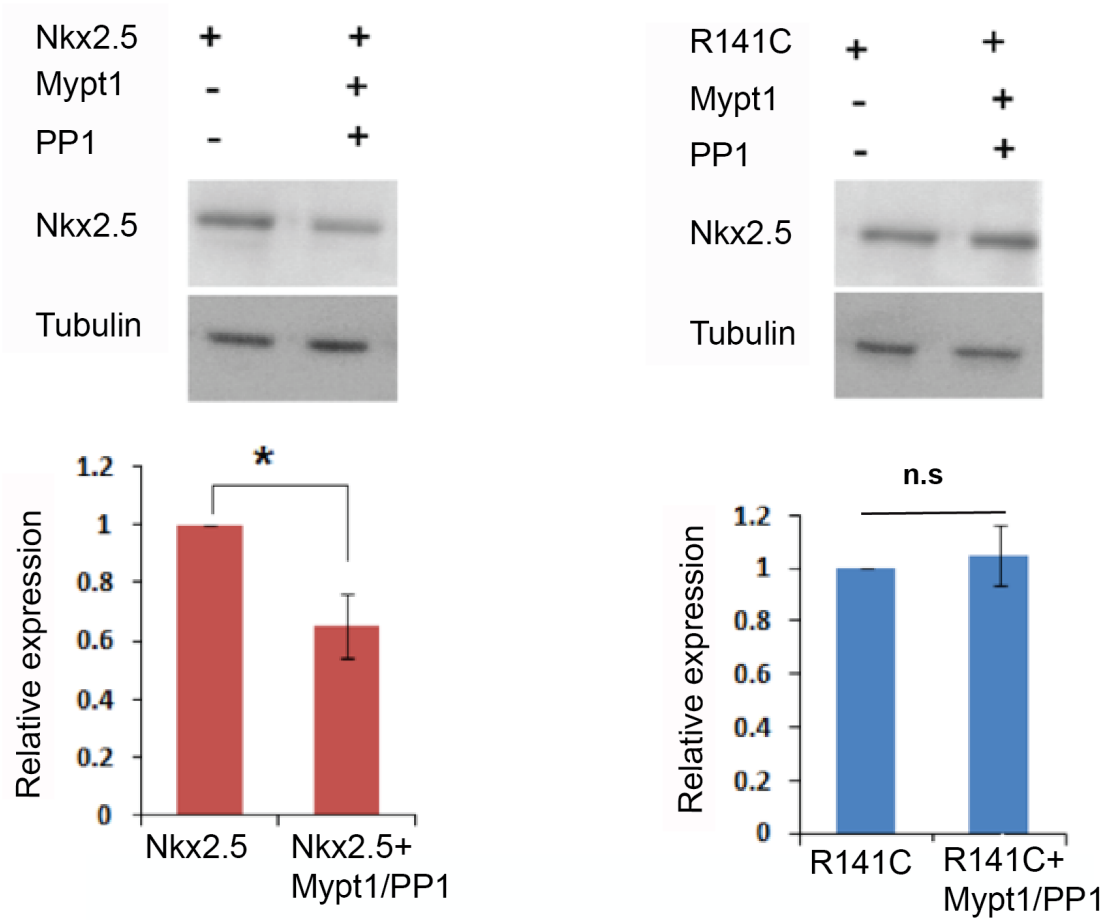
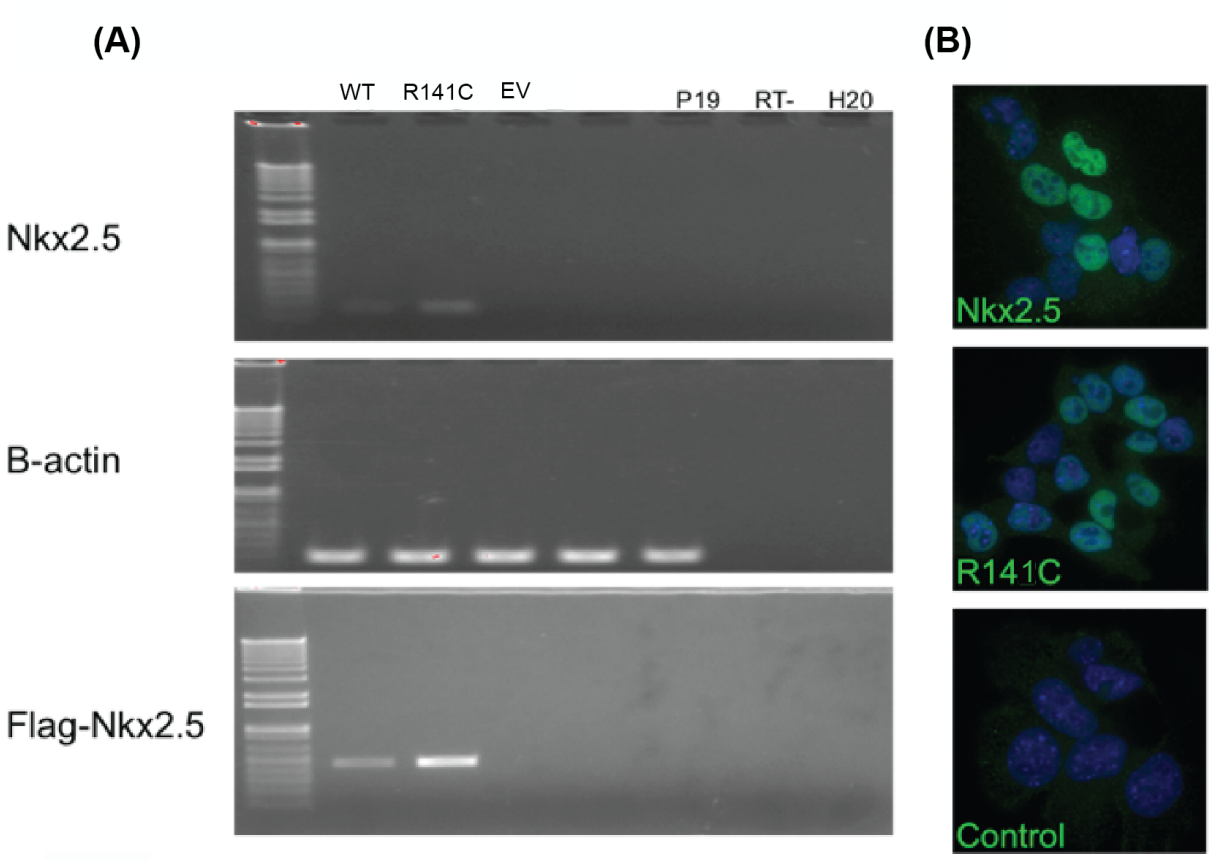


Figure 4.3: The presence of Mypt1/PP1 reduced the WT Nkx2.5 but not R141C mutant protein level.

P19 cells were transiently transfected with Flag-Nkx2.5 or Flag-R141C, with or without HA-PP1 and myc-Mypt1. After 24 hours of transfection, total protein was harvested and analyzed by western blots using antibodies specific to Nkx2.5. α-Tubulin antibody was used as a loading control. Data were expressed relative to Nkx2.5 or R141C alone and band densities were quantified using imageJ, n.s not significant, *p<0.05, n=4,

4.4.4 Both Nkx2.5 and R141C protein interact with endogenous Mypt1 and PP1 in P19 cells

The observed resistance of the R141C mutant to the MP-induced exclusion and protein degradation implies a possible loss of interaction between the R141C mutant protein and the MP complex. In order to investigate the interaction between R141C protein and Mypt1/PP1 proteins, we performed co-immunoprecipitation studies. First, we generated three stable P19 cells overexpressing Flag WT Nkx2.5, Flag R141C, and empty vector EV as a negative control. RT-PCR and immunofluorescence analysis confirmed the overexpression of Flag WT-Nkx2.5 and Flag R141C mRNA and protein, respectively, (Figure 4.4 A-B). Protein lysates from these cells were collected and incubated with Flag beads for immunoprecipitation. Western blots were then performed using Nkx2.5, Mypt1, and PP1 antibodies. Results showed that Nkx2.5 and R141C protein interacted with both endogenous Mypt1 and PP1 in P19 cells (Figure 4.4 C).


(A)
WT
R141C
EV
P19
RT-
H20
Nkx2.5
B-actin
Flag-Nkx2.5
(B)
Nkx2.5
R141C
Control

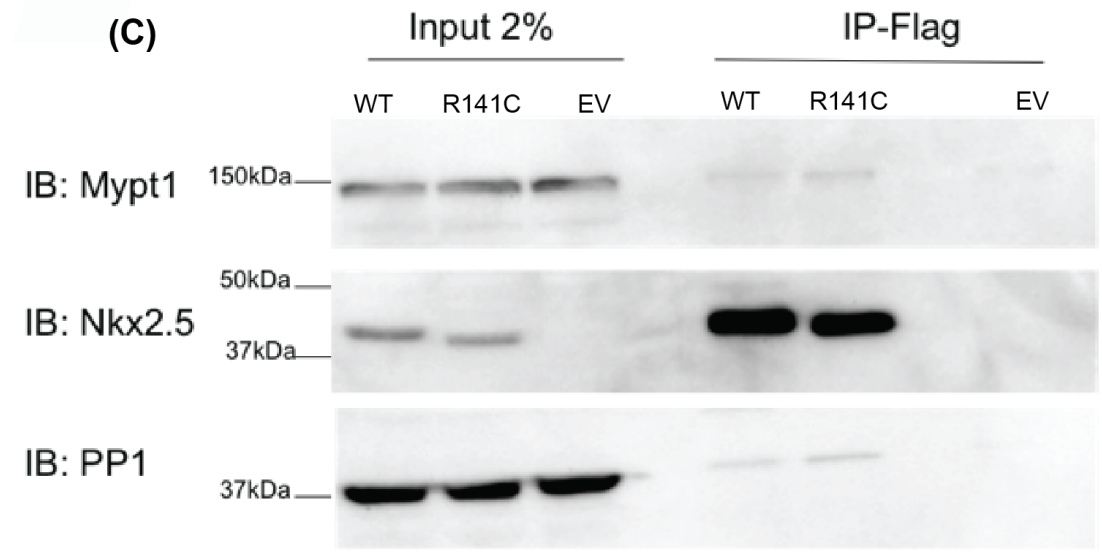

(C)
Input 2%
IP-Flag
WT
R141C
EV
WT
R141C
EV
IB: Mypt1
150kDa
IB: Nkx2.5
50kDa
37kDa
IB: PP1
37kDa

Figure 4.4: Endogenous Mypt1 and PP1 interact with WT Nkx2.5 and R141C mutant in stable P19 cells.

(A) mRNA was collected from stable P19 cells overexpressing Flag WT Nkx2.5 (WT), Flag R141C (R141C), empty vector (EV), and non transfected P19 cells. RT-PCR was performed using Nkx2.5, Flag Nkx2.5 and B-actin primers. RT- and H2O served as negative controls. **(B)** Nkx2.5 and R141C proteins were expressed in the cells. Stable P19 cells overexpressing Nkx2.5, R141C, and control were fixed and stained with anti-Nkx2.5 antibody, and the nucleus was stained with DAPI. **(C)** Total protein was harvested from stable cells and immunoprecipitated with Anti-Flag antibody coated beads. Immunoprecipitated lysates and input fractions were analyzed by western blot with the antibodies indicated in the figure.

4.5 Discussion

In this report, we studied the protein-protein interaction between Mypt1/PP1 and R141C mutation using co-Immunoprecipitation. We also studied the effects of this interaction on the transcriptional activity, subcellular localization and protein levels of the R141C mutant as compared to the WT Nkx2.5. Since the R141C mutation is located in the first amino acid of the potential PP1-binding motif (RVLF) in the Nkx2.5 protein, we tested whether the mutant protein binds to Mypt1/PP1. Our results showed that both the WT Nkx2.5 and R141C mutant protein bind to endogenous Mypt1/PP1 in P19 cells. These data suggest that it is not likely that WT Nkx2.5 protein binds to MP complex through the RVxF motif, or that there could be another site for this interaction. It is also possible that there is an indirect interaction of the WT Nkx2.5 protein with the MP complex that involves other proteins. However, we showed that the interaction of the R141C mutant protein with the MP complex did not have the same effect on the R141C transcriptional activity and subcellular localization as it did for the WT Nkx2.5.

It is also possible that Nkx2.5 binds to Mypt1 protein but not PP1; further experiments are needed to answer this question. We also found out that it is not likely that Nkx2.5 binds to Mypt1/PP1 through the RVxF motif that is located in the HD for several reasons. One reason is that the RVxF motif is located randomly in about 25% of all proteins that are not necessarily bound to PP1 (22, 23).The second reason is that this motif is located in a globular domain, HD, which is unlikely to be the site of PP1 interaction (24).

- This report highlights an important observation: that the R141C mutant does not respond similarly to the WT Nkx2.5 in the presence of the MP complex. In contrast with WT Nkx2.5, the R141C mutant showed resistance to the MP complex mediated inhibition of the WT Nkx2.5 transcriptional activity, exclusion of WT Nkx2.5 protein to the perinuclear region, as well as reduce protein level. The mechanism of this action is not clear yet and further experiments need to be performed to investigate the interaction between Nkx2.5 and Mypt1/PP1. The interaction of Nkx2.5 with MP is complex and It could involve multiple sites of interaction and Does not fully require the Arginine in the RVxF motif in Nkx2.5 protein.

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CHAPTER 5

Discussion

Chapter 5 discusses the overall findings of my doctoral thesis. First, we modeled the human CHDs heterozygous associated mutation *R142C*, using the mESCs. Second, we generated and characterized the heterozygous mouse *Nkx2.5*$^{R141C/+}$ and showed that it is a novel mouse model for studying CHDs. Finally; we examined the interaction of the R141C mutant protein with the Mypt1/PP1 complex.

5.1 Modeling the human congenital heart defects using mESCs (Figure 5.1 A)

Despite improvements on understanding the stages of normal heart development and the regulatory gene networks involved in this process, the etiology of CHDs is still unknown (1). mESCs have the capacity to differentiate into cardiomyocytes and recapitulate the early stages of the developing embryo (2). Therefore, modeling the CHDs using mESCs is a good tool for understanding the early molecular mechanism of the CHDs. In Chapter 2, we used a homologous recombination knock-in gene strategy to generate a mESC harboring a human CHD causing mutation *NKX2.5 R142C*. We investigated the ability of the *Nkx2.5*$^{R141C/+}$ mESCs to modulate cardiomyogenesis. We also examined the subcellular localization of Nkx2.5 protein in the *Nkx2.5*$^{R141C/+}$ mESCs and the defects that occurs in gene expression during early *in vitro* cardiomyogenesis.

The *Nkx2.5*$^{R141C/+}$ mESCs showed lower percentage of cardiac muscle formation and beating EBs than the control *Nkx2.5*$^{+/+}$ cells during *in vitro* cardiomyogenesis. A previous study also showed that the early stage of cardiomyogenesis in *Nkx2.5*$^{EGFP/+}$ cells was significantly reduced and delayed (3). Another study analyzed cardiomyogenesis at later stages of EB differentiation (day 17) from *Nkx2.5*$^{+/-}$ and *Nkx2.5*$^{-/-}$ cells and found that cardiac differentiation was not perturbed (4). In this study, we did not examine the effect of the R141C heterozygous mutation at late stages of cardiomyogenesis. Therefore, the reduced cardiomyogenesis at early stages in the *Nkx2.5*$^{R141C/+}$ mESCs could be transient and it could be compensated for at later stages of cardiomyogenesis.

We also found an impairment of Nkx2.5 nuclear localization during cardiomyogenesis in *Nkx2.5*$^{R141C/+}$ mESCs and in HL1 cardiac cells. Overexpression of the R141C mutant plasmid in HL1 cardiac cells showed that around 30% of cells express R141C protein in both the nucleus and the cytoplasm, whereas the WT Nkx2.5 was mostly nuclear. We also found that the R141C mutant protein is resistant to treatment with leptomycin B, an inhibitor of nuclear export, because it did not accumulate in the nucleus even after blockage of nuclear protein export. This suggests that the R141C protein fails to enter the nucleus, and thus the R141C mutation affects nuclear import in HL1 cells. However, the R141C mutant protein was mostly nuclear when overexpressed in non-cardiac cells COS-7 (5) and P19 (in Chapter 4).

The transcriptional activity of the R141C mutant was low compared to WT Nkx2.5 in COS-7 (5) and P19 cells. Therefore, we suggest that reduced cardiomyogenesis in *Nkx2.5* $^{R141C/+}$ mESCs *in vitro* is not only the result of impairment in nuclear localization of the R141C protein but could be also because of the weak DNA binding, lower transcriptional activity and weak interaction of the R141C protein with cardiac transcription factors as shown in the previous study (5).

It is also known that various signaling pathways modulate the function of Nkx2.5 during cardiomyogenesis, which could be absent in the non-cardiac cells. For example, Wnt signaling was shown to play a role in modulating Nkx2.5 subcellular localization during cardiac differentiation (6). Therefore, the different cell contexts and signaling pathways involved might explain the discrepant results in the localization of the R141C mutant protein between the cardiac and non-cardiac cells. A recent study identified a second nuclear localization signal (NLS) in the C-terminus of the Nkx2.5 protein. This finding could explain the fact that we still see some nuclear R141C protein during cardiomyogenesis and when overexpressed in cardiac and non-cardiac cells.

We also reported the global changes of gene expression in *Nkx2.5*$^{R141C/+}$ mESCs at day 6 of cardiomyogenesis. The microarray analysis of day 6 of cardiomyogenesis in *Nkx2.5* $^{R141C/+}$ revealed 366 disregulated genes when compared to control cells. These genes were found to be involved in heart development, cardiac chamber morphogenesis, cardiac progenitor differentiation and extracellular matrix organization (see Figure 3.5, Table B). Also, using our

microarray analysis and data from previous ChIP-seq analysis from a E11.5 developing embryo, revealed list of genes that have in their promoters the hybrid DNA sequence motif bound by Nkx2.5 and Meis1(7). Interestingly, Nkx2.5 and Meis1 are known to be important for the conduction system and genome wide studies in humans have shown that mutations in Nkx2.5 and Meis1 are associated with conduction defects (8). Patients with the heterozygous *R142C* mutation displayed a conduction abnormality manifested by progressive AV block (9) and our identification of misregulated genes that are important for the conduction system in the *Nkx2.5*$^{R141C/+}$ mESCs could help understand the pathogenesis of these CHDs. The patients with the *R142C* mutation also displayed ASD, which arises from a defect that occurs during EC formation during heart development (9). In the *Nkx2.5*$^{R141C/+}$ cells, we found some downregulated genes that are important for the formation of EC during heart development. Therefore, *Nkx2.5*$^{R141C/+}$ mESCs provide us with subsets of genes that could be disregulated during early heart development in patients with the *R142C* heterozygous mutation.

5.2 *Nkx2.5*$^{R141C/+}$ is a novel mouse model to study human congenital heart defects (Figure 5.1 B)

In humans, around 50 heterozygous mutations in *NKX2.5* are found to be associated with CHDs (10). These mutations are segregated in families as an autosomal dominant mutation. The majority of patients with *NKX2.5* mutations display AV block and a secundum ASD. Due to the difficulties in accessing

human heart tissues, it is hard to study the molecular mechanism of CHDs caused by mutations in Nkx2.5 or other cardiac transcription factors. A mouse model that harbors a human CHD mutation is a good tool for studying the pathogenesis caused by these mutations.

In Chapter 3, we showed the generation and characterization of a novel mouse model with a knock-in R141C mutation in Nkx2.5 harboring a human CHD. A mouse model allowed us to study the functional defect *in vivo* and test the ability of the mutation to cause the disease.

The intercrosses of the *Nkx2.5*$^{R141C/+}$ heterozygous mice showed an unexpected Mendelian ratio in the born mice. The homozygous embryos *Nkx2.5*$^{R141C/R141C}$ were never born, indicating that the R141C homozygous mutation causes embryonic lethality. The homozygous embryos showed growth retardation at E10.5. Therefore, we suggest that the R141C mutation affects the function of Nkx2.5 during heart development between E7.5 and E9.5.

Histological examinations of the E9.5 and E10.5 *Nkx2.5*$^{R141C/R141C}$ embryos showed that the heart was formed. However, the hearts of the *Nkx2.5*$^{R141C/R141C}$ embryos showed delayed heart morphogenesis, including the absence of EC formation. Also, the expression of genes important for EMT were significantly downregulated in the *Nkx2.5*$^{R141C/R141C}$ hearts compared to control. These defects in the homozygous heart can be explained in part that Nkx2.5 plays a role in both the myocardium and endocardium lineages. It has been shown that Nkx2.5 is expressed in the cardiac crescent stage and that

myocardial and endocardial cells arise from common progenitor cells located in the cardiac crescent (11). A previous study also performed transcriptome-wide expression profiling on fluorescence activated cell sorting (FACS) using a Nkx2.5 enhancer, isolating cells from the cardiac crescent, linear heart tube and during heart looping stages and found that GFP positive cells expressed both myocyte and endothelial differentiation markers (12). Another study found that the regulation of Etsrp71 in the endocardium is under the direct control of Nkx2.5 (13). The Etsrp71 protein is also known to downregulate Tie2, which is expressed in the endothelial progenitor (13, 14). These results support the notion that Nkx2.5 is involved in endocardial/endothelial specifications.

The hearts of the *Nkx2.5*$^{R141C/R141C}$ embryos also showed an absence of ventricular trabeculation, which was already evident in the controls. The expression of chamber specific genes such as *Anf*, *Mlc2v* and *Cx40* were low indicating poor cardiac specification caused by R141C homozygous mutation. However, in the homozygous heart, the mRNA level of the *Mhc7* showed comparable level to that of the controls, indicating that cardiomyocyte differentiation was compensated during heart development in *Nkx2.5*$^{R141C/R141C}$ embryos. The defects we observed in our *Nkx2.5*$^{R141C/R141C}$ embryos are similar to the phenotypes observed in the null Nkx2.5 embryos (15, 16). We showed that the Nkx2.5 protein level in the heart of the *Nkx2.5*$^{R141C/R141C}$ embryos is similar to the WT and heterozygous embryos and was mostly localized in the nucleus. Our findings show that the R141C homozygous mutation causes a decrease in the

function of Nkx2.5, which is evident by the downregulation of Nkx2.5 downstream genes such as *Anf, Mlc2v, Cx40* and *Actc1*.

Previous studies have shown that *Nkx2.5* heterozygous mice are born grossly normal but with CHDs and conduction defects (17–20). We also showed that the *Nkx2.5*$^{R141C/+}$ heterozygous mice were grossly normal, but showed cardiac and conduction defects. Therefore, one WT allele of the Nkx2.5 gene in the heterozygous mice is able to form a complete but not healthy heart. We also studied Nkx2.5 downstream target genes in the hearts of the *Nkx2.5*$^{R141C/+}$ embryos. Among them, *Anf* and *Mlc2v* were significantly downregulated compared to controls. *Anf* and *Mlc2v* play an important role during ventricle chamber morphogenesis (21–23). Therefore, the alteration of gene expression in our heterozygous mouse model could affect the tightly regulated gene network during heart development leading to various CHD phenotypes and conduction defects (24, 25).

Previous studies showed that *Anf* was downregulated in the null Nkx2.5 homozygous embryos (15) but not in the *Nkx2.5*$^{+/-}$ embryos (26). In contrast, in the *Nkx2.5*$^{R141C/+}$ embryo hearts, we find that the *Anf* and *Mlc2v* mRNA levels were significantly downregulated. The expression of *Anf* was downregulated in our mouse model *Nkx2.5*$^{R141C/+}$ but was not affected in the *Nkx2.5*$^{+/-}$ heterozygous mice (26). This result indicates that the point mutation R141C in the *Nkx2.5*$^{R141C/+}$ embryo hearts could have a broader effect than the null allele in the *Nkx2.5*$^{+/-}$ heterozygous mice. However, in the newborn hearts of the

Nkx2.5$^{R141C/+}$ mice, the mRNA level of *Anf* continued to be downregulated but not *Mlc2v,* which was comparable to control newborn hearts. These results suggest a compensatory mechanism during heart development for *Mlc2v* but not for *Anf.* Moreover, the mRNA levels of the other Nkx2.5 downstream targets *Cx40* and *Actc1* as well as endothelial genes such as *Estrp71* in the *Nkx2.5*$^{R141C/+}$ embryo hearts were similar to controls. It is possible that the normal allele of Nkx2.5 is enough to activate these genes or that other transcription factors compensate for some Nkx2.5 function in order to obtain normal expression levels of its direct or indirect target genes. It is also possible that these genes could be misregulated before or after E9.5, which we did not examine in this study.

Histological and gross examination of the heterozygous mice showed variable CHD phenotypes including ASD, VSD, ventricle trabeculation and DORV. Patients with a *R142C* heterozygous mutation also displayed various CHD phenotypes such as ASD, VSD, TOF (9). Germline mutations in the *NKX2.5* gene lack genotype–phenotype correlation, meaning that the same mutation is associated with diverse CHDs, or the same CHD is found in patients with different Nkx2.5 mutations (24).

Most patients with *R142C* mutations also displayed a conduction system defect in which most of them (12/13) suffered from progressive AV block (9). We show that *Nkx2.5*$^{R141C/+}$ heterozygous mice also displayed conduction defects. The expression level of the ion channel genes that are important for cardiac conduction and contraction were altered in the *Nkx2.5*$^{R141C/+}$ newborn mice. The

defect in the conduction system of the heterozygous mice could be due to defects that occur during the development of specialized conduction cells in which Nkx2.5 is known to play an important role (27–29). For example, during early heart development, *Nkx2.5, Tbx5* and *Id2* function in the specification of the myocytes in the conduction system (29). Several studies also showed that the *NKX2.5* gene regulates expression of important genes involved in conduction and contraction at both perinatal and postnatal stages (28, 30, 31). Therefore any disruption of the conduction system program leads to defects later on in adulthood. We reported a high penetrance of prolonged AV block in the *Nkx2.5*$^{R141C/+}$ adult heterozygous mice, replicating the conduction defects seen in human patients with *R142C* heterozygous mutations.

In Chapter 2 and Chapter 3, we showed the defects associated with the R141C mutation using *in vitro* and *in vivo* approaches. We showed that the *Nkx2.5*$^{R141C/+}$ mESCs displayed reduced cardiomyogenesis early when compared to *Nkx2.5*$^{+/+}$ mESCs. In contrast, *Nkx2.5*$^{R141C/+}$ heterozygous mice did not show reduced cardiomyogenesis compared to WT mice. These conflicting results could be explained in part by the fact that a compensatory mechanism might occur during cardiac differentiation in the heart of the heterozygous mice when compared to cardiomyocytes in a dish. The reduction in cardiomyogenesis *in vitro* is noted as early as days 6 and 9. The cardiac progenitors at days 6 and 9 *in vitro* reflect the cardiac progenitors in the cardiac crescent and the linear heart tubes at early stages of heart development *in vivo* (7.5 - 8.5). However, we did not examine E7.5 - 8.5 *Nkx2.5*$^{R141C/+}$ heterozygous embryos. Therefore, the

defect that occurs at an early stage *in vitro* could be transient and could be compensated at later stages of cardiomyogenesis, as we see at E9.5 *in vivo*. *Anf* and *Mlc2v* mRNA levels were downregulated *in vitro* and *in vivo*, indicating a direct effect of R141C mutation on the activation of these two genes.

Furthermore, we found an impairment of nuclear localization of Nkx2.5 protein during cardiomyogenesis in the heterozygous mESCs and HL1 cardiac cells. In contrast, we showed that Nkx2.5 is mostly nuclear in the myocardium of both heterozygous and homozygous E9.5 embryos. However, we cannot rule out the possibility that there could be an impairment of nuclear localization of the mutant protein that could occur transiently at an early stage before E9.5 that we did not examine *in vivo*.

5.3 Interaction of the R141C mutant with Mypt1/PP1 (Figure 5.1 C)

A previous study in our lab showed that Mypt1/PP1 are novel binding partners of Nkx2.5 during cardiomyogenesis *in vitro* (6). In Chapter 4, we further examined the R141C mutation's ability to impact binding to Mypt1/PP1 complex. Nkx2.5 contains a putative consensus PP1- binding sequence, RVxF, located in the N-terminus of the homeodomain (HD) (6, 32). Notably, this sequence is mutated in patients with the *R142C* heterozygous mutation, resulting in the substitution of the first amino acid in the motif from arginine to cysteine.

We studied protein-protein interactions by performing Co-IP and found that both WT Nkx2.5 and R141C protein binds to endogenous Mypt1 and PP1

proteins. However, while the transcriptional activity of the WT Nkx2.5 gene is inhibited in the presence of Mypt1/PP1, the R141C mutant transcriptional activity remains unaffected in the presence of Mypt1/PP1. Moreover, the WT Nkx2.5 protein was excluded to the perinuclear region in the presence of Mypt1/PP1, but the R141C protein was mostly nuclear in the presence or absence of Mypt1/PP1. We also showed that the exclusion of the WT Nkx2.5 to the perinuclear region was associated with reduced WT Nkx2.5 protein level. However, the R141C protein level was not affected in the presence of Mypt1/PP1.

It is still not clear how Mypt1/PP1 interacts with R141C protein. The presence of Mypt1/PP1 did not have the similar effect on the R141C transcriptional activity and cellular localization as it did for the WT Nkx2.5. We performed Co-IP and found that both WT Nkx2.5 and R141C mutation bind to endogenous Mypt1 and PP1. The Co-IP experiment, however, does not discriminate between direct and indirect binding of R141C protein with Mypt1/PP. It is also possible that Nkx2.5 binds to Mypt1 protein but not PP1; further experiments are needed to address this question. We also found that it is not likely that Nkx2.5 binds to Mypt1/PP1 through the RVxF motif that is located in the HD for multiplel reasons. One reason is that the RVxF motif is located randomly in about 25% of all proteins that are not necessarily bound to PP1 (33, 34).The second reason is that this motif is located in a globular domain, HD, which is unlikely to be the site of PP1 interaction (35).

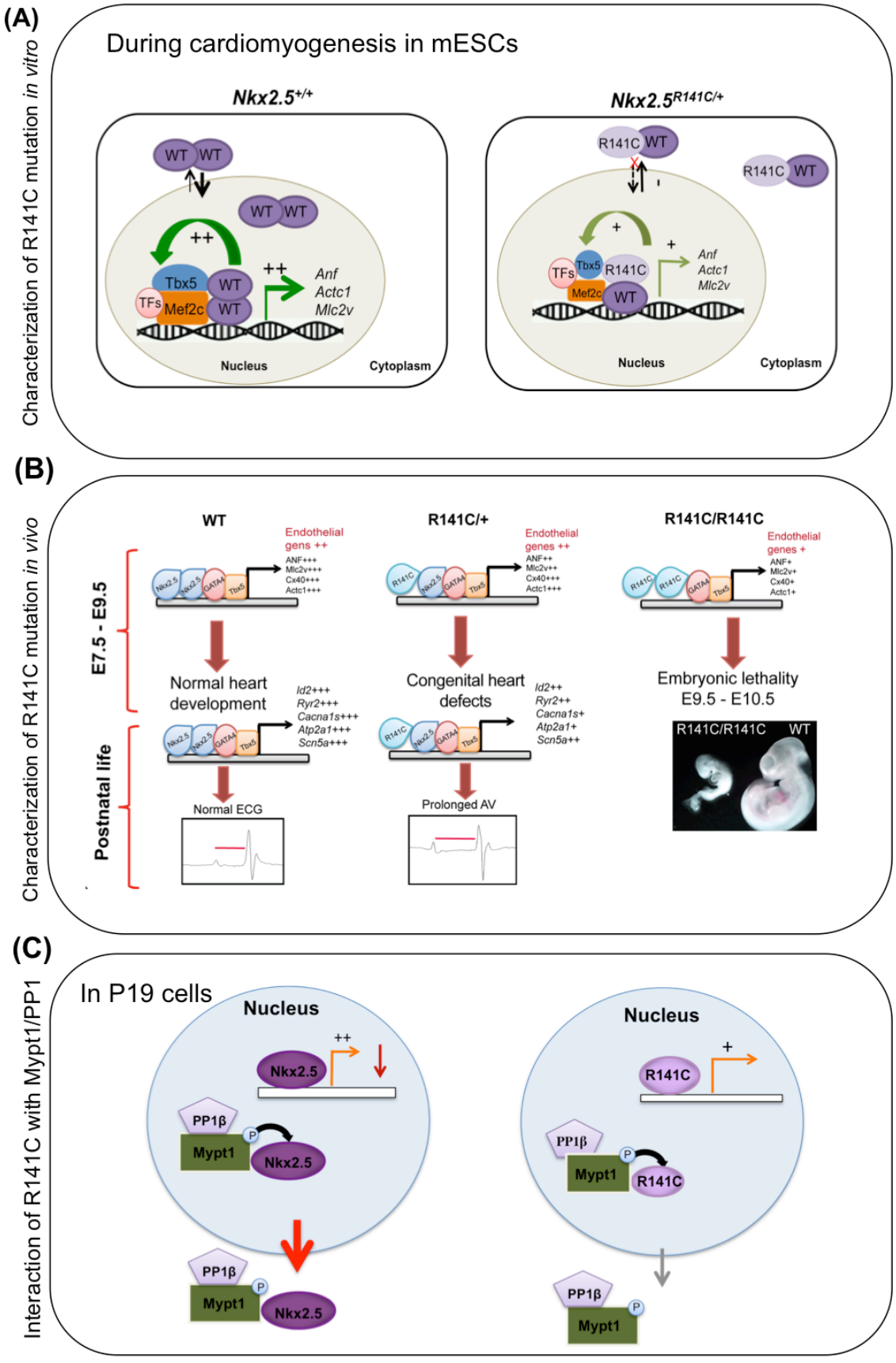
(A)
Characterization of R141C mutation in vitro
During cardiomyogenesis in mESCs
Nkx2.5+/+
Nkx2.5R141C/+
WT WT
R141C WT
Tbx5
TFs
Mef2c
++
+
Anf
Actc1
Mlc2v
Nucleus
Cytoplasm
(B)
Characterization of R141C mutation in vivo
WT
R141C/+
R141C/R141C
Endothelial genes ++
Endothelial genes +
ANF+++ Mlc2v+++ Cx40+++ Actc1+++
ANF++ Mlc2v++ Cx40+++ Actc1+++
ANF+ Mlc2v+ Cx40+ Actc1+
E7.5 - E9.5
Postnatal life
Normal heart development
Congenital heart defects
Embryonic lethality E9.5 - E10.5
Id2+++ Ryr2+++ Cacna1s+++ Atp2a1+++ Scn5a+++
Id2++ Ryr2++ Cacna1s+ Atp2a1+ Scn5a++
Normal ECG
Prolonged AV
R141C/R141C WT
(C)
Interaction of R141C with Mypt1/PP1
In P19 cells
Nucleus
Nkx2.5
PP1β
Mypt1
P
R141C

Figure 5.1 Summary and proposed models of my PhD findings.

(A) Proposed model of the *in vitro* cardiomyogenesis in *Nkx2.5*$^{+/+}$ and *Nkx2.5*$^{R141C/+}$ mESCs. *Nkx2.5*$^{R141C/+}$ mESCs show reduced cardiomyogenesis and lower expression of Nkx2.5 downstream targets, including several endothelial and conduction genes. *Nkx2.5*$^{R141C/+}$ mESCs also show impaired nuclear localization, which results in lower Nkx2.5 protein dosage in the nucleus. **(B)** Proposed model of the *Nkx2.5*$^{+/+}$, *Nkx2.5*$^{R141C/+}$ and *Nkx2.5*$^{R141C/R141C}$ mice during early stages of heart development and postnatal life. *Nkx2.5*$^{+/+}$ mice show normal gene expression of Nkx2.5 target genes and genes important for heart development and resulted in normal heart morphogenesis and conduction system. *Nkx2.5*$^{R141C/+}$ mice show low expression of some Nkx2.5 downstream targets such as *ANF* and *Mlc2v* at early stages of heart development. *Nkx2.5*$^{R141C/+}$ mice also display CHDs. In postnatal life, *Nkx2.5*$^{R141C/+}$ mice show downregulation of ion channel genes which results in progressive AV block later on in adulthood. **(C)** Proposed model of the interaction between the WT Nkx2.5 and the R141C mutant protein with Mypt1/PP1 complex. Both the WT Nkx2.5 and R141C interact with Mypt1/PP1. The presence of Mypt1/PP1 resulted in the inhibition of the WT Nkx2.5 transcriptional activity and exclusion to the perinuclear region. However, the transcriptional activity of the R141C mutant remain the same and is not excluded to the perinuclear region in the presence of Mypt1/PP1. The mechanism of interaction between the R141C mutant protein and Mypt1/PP1 remains to be determined.

5.4 Conclusion and Future Directions

In the current study, we characterized and examined the function of the R141C mutation during cardiomyogenesis in mESCs *in vitro* and the CHDs associated with the R141C mutation using an *in vivo* mouse model. We thought that the reduced cardiomyogenesis *in vitro* could be transient. In order to test this possibility, *Nkx2.5*$^{R141C/+}$ mESCs could be further differentiated until day 21 and then the percentage of cardiac muscle formation and gene expression of cardiac muscle genes could be further analyzed.

The generation of *Nkx2.5*$^{R141C/+}$ heterozygous mice provides a model to study CHDs and that will assist the understanding of the genetic mechanisms underlying the embryological basis of septation and conduction defects. Consistent with the cardiac phenotype seen in humans with the *R142C* mutation, the heart of the *Nkx2.5*$^{R141C/+}$ mice also display morphological and conduction defects. It is interesting that the heterozygous mutation is sufficient to form a complete heart but is insufficient to make a healthy heart. In the homozygous embryos, the R141C mutant protein may have functional deficits in the endocardium and myocardium lineages, which are shown by the absence of EMT in the EC and improper development of the ventricle. Further analysis is needed to examine the ability of the R141C mutation to bind and activate the known Nkx2.5 targets. It is also possible that the R141C mutation has different target genes than the WT Nkx2.5 and this can be tested by performing ChIP-seq analysis, which will allow for the identification of sets of direct targets of Nkx2.5

and R141C mutation. It would be also useful to test the changes that could occur in gene expression at the cardiac crescent and the linear heart tube stage in the homozygous and heterozygous embryos and compare it with WT embryo, which can be tested by performing whole mount in situ hybridization on Nkx2.5 downstream genes and some endothelial genes.

In conclusion, our *Nkx2.5*$^{R141C/+}$ mESC represents a novel *in vitro* model of human CHDs. However, *in vitro* mESCs gave similar but non-identical results compared to the *in vivo* results. Therefore, any study on patient-derived iPS cells would need to take into consideration that the *in vitro* differentiation of ES cells might not be perfect. Both *Nkx2.5*$^{R141C/+}$ mESC and *Nkx2.5*$^{R141C/+}$ mice could provide models for discovering new treatments and allow researchers to better understand the pathology of CHDs.

5.5 References

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