Role Of Nitric Oxide In Embryonic Heart Development And Adult Aortic Valve Disease

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Graduate Program in Pharmacology and Toxicology
A thesis submitted in partial fulfillment of the requirements for the degree in Doctor of Philosophy
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ROLE OF NITRIC OXIDE IN EMBRYONIC HEART DEVELOPMENT AND
ADULT AORTIC VALVE DISEASE

(Thesis format: Integrated Article)

by

Yin Liu

Graduate Program in Pharmacology

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

The School of Graduate and Postdoctoral Studies
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Abstract

Congenital heart disease (CHD) is the most common birth defect in infants. Identifying factors that are critical to embryonic heart development or CHDs in general could further our understanding of the disease and may lead to new strategies of its prevention and treatment. Endothelial nitric oxide synthase (NOS3/eNOS) is known for many important biological functions including vasodilation, vascular homeostasis and angiogenesis. Previous studies have shown that deficiency in NOS3 results in congenital septal defects, cardiac hypertrophy and postnatal heart failure. In addition, NOS3 is pivotal to morphogenesis of aortic valve and myocardial capillary development. The aim of my thesis was to investigate the role of NOS3 in the embryonic and adult heart. I discovered that NOS3 deficiency resulted in coronary artery hypoplasia in fetal mice and spontaneous myocardial infarction in postnatal hearts. Coronary artery diameters, vessel density and volume were significantly decreased in NOS3⁻/⁻ mice at postnatal day 0. Lack of NOS3 also down-regulated the expression of Gata4, Wilms tumor-1, vascular endothelial growth factor, basic fibroblast growth factor and erythropoietin in the embryonic heart at E12.5, and inhibited migration of epicardial cells into the myocardium. In addition, my data show that the overall size and length of mitral and tricuspid valves were decreased in NOS3⁻/⁻ compared with WT mice. Echocardiographic assessment showed significant regurgitation of mitral and tricuspid valves during systole in NOS3⁻/⁻ mice. Immunostaining of Snail1 was performed in the embryonic heart. Snail1 positive and total mesenchymal cells in the AV cushion were decreased in NOS3⁻/⁻ compared with WT mice at E10.5 and E12.5. Finally, in the adult aortic valves, NOS3 is important in inhibition of thrombosis formation. Deficiency in NOS3 leads to aortic valve thrombosis.
and calcification. At 12 months old, 72% (13/18) of $NOS3^{-/-}$ mice showed severe spontaneous aortic valve thrombosis compared with WT mice (0/12). \textit{Ex vivo} culture of aortic valves showed that platelet aggregation and adhesion were significantly increased in $NOS3^{-/-}$ aortic valves compared with WT aortic valves. There was also a significant regurgitation of the aortic valve during systole in the $NOS3^{-/-}$ compared with WT mice. In addition, $NOS3$ deficiency resulted in significant aortic valve stenosis, calcification and fibrosis. In summary, these data suggest NOS3 plays a critical role in embryonic heart development and morphogenesis of coronary arteries and inhibits thrombosis formation in the adult aortic valves.
Co-Authorship Statement

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

Dr. Qingping Feng contributed to experimental design, data interpretation, and manuscript preparation for all experiments. Drs. Robert E. Poelmann, Adriana C. Gittenberger-de Groot, Jeffrey Robbins contributed to manuscript preparation for Chapter 2. Additionally, Dr. Sharon Lu assisted with the troubleshooting in all the experiments. Mrs. Murong Liu coordinated the animal breeding and genotyping.

Chapter 2: Dr. Fuli Xiang performed the EPDC cultures and western blot analysis of hu-NOS3 expression (Figure 2.1 and 2.8).

Chapter 3: Dr. Lu Man assisted with the echocardiography imaging (Figure 3.5, 3.6 and 3.7). Dr. Fuli Xiang helped with the endocardial cushion explants. (Figure 3.10)

Chapter 4: Dr. Fuli Xiang helped with echocardiography imaging. (Figure 4.3, 4.4).
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List of abbreviation, symbols and nomenclature

3D: Three dimensional
Ad-GFP: adenoviral green fluorescent protein construct
Ad-LacZ: adenoviral LacZ construct
Ad-NOS3: adenoviral nitric oxide synthase-3 construct
ALS: Amyotrophic Lateral Sclerosis
ANP: Atrial natriuretic peptide
ARS: Axenfeld-Rieger syndrome
ASD: Atrial septal defect
AV: Atrioventricular
BMP-2: Bone morphogenetic protein-2
BrdU: bromodeoxyuridine
CAVS: Calcific aortic valve stenosis
CHD: Congenital heart disease
DAB: diaminobenzidine tetrahydrochloride
DETA-NO: Diethylenetriamine NONOate
E: Embryonic
ELISA: enzyme-linked immunosorbent assay
EMT: Epithelial to mesenchymal transition
EndMT: Endocardial to mesenchymal transition
EPDC: Epicardial-derived cells
EPO: Erythropoietin
EPOR: Erythropoietin receptor
FGF: Fibroblast growth factor
GFP: Green fluorescent protein
H/E: Hematoxylin/eosin
HCAD: Hypoplastic coronary artery disease
IGDS: Iridogoniodygenesis syndrome
KLF2: Krüppel-like factor 2
LCA: Left coronary artery
L-NAME: N⁴G-nitro-L-arginine methyl ester
LV: Left ventricular
LVIDd: LV internal end-diastolic dimension
LVIDs: LV internal systolic dimension
Mef2C: Myocyte-specific enhancer factor 2
MHC: Myosin heavy chain
MMLV: moloney murine leukemia virus
NF-κB: nuclear factor kappa B
NO: Nitric oxide
NOS1: Nitric oxide synthase isoform 2 or neuronal nitric oxide synthase
NOS2: Nitric oxide synthase isoform 2 or inducible nitric oxide synthase
NOS3: Nitric oxide synthase isoform 3 or endothelial nitric oxide synthase
P0: Postnatal day 0
PBS: Phosphate buffered saline
PCR: Polymerase chain reaction
PEO: Pro-epicardial organ
PI3K: Phosphatidylinositol-3 kinase
PKB: Protein kinase B
PKG: Protein kinase G
RCA: Right coronary artery
ROS: Reactive oxygen species
RV: Right Ventricle
SNO: S-nitrosothiol
Tgfβ: Transforming growth factor beta
TTC: Triphenyltetrazolium chloride
VEGF: Vascular endothelial growth factor
VHD: Valvular heart disease
VICs: Valve interstitial cells
VSD: Ventricular septal defect
WT: Wildtype
Wt1: Wilm’s tumor -1
α-SMA: α-smooth muscle actin
μ was used for micro, α was used for alpha, and β was used for beta.
*, † and ‡ are used for denoting statistical significance.
1 CHAPTER 1

Literature Review

1.1 Congenital Heart Disease

Congenital heart disease refers to a defect in the structure of the heart and/or vessels present at birth. This defect, however, can be detected earlier by prenatal diagnosis such as fetal echocardiography. The disease affects up to 5% of people of the general population and is the leading cause of death in infants during the first year of life in the industrialized countries.\textsuperscript{1, 2} It is estimated that 96,000 adults in Canada have congenital heart disease.\textsuperscript{3} These patients have increased risk of arrhythmia, bacterial endocarditis and heart failure later in life.\textsuperscript{4} Thus, congenital heart disease is a major cause of mortality and morbidity in both infants and adults. The cause of congenital heart disease may be either genetic or environmental, but is usually a combination of both. It is estimated that only 15% of all congenital heart disease can be traced to a known cause.\textsuperscript{4} Some genetic factors have been linked to cause congenital heart disease, while the majority of factors are still unknown. Thus understanding the mechanisms that regulate the formation of the heart and the coronary artery system could further our understanding of the disease and lead to new strategies of its prevention or treatment.

1.2 Valvular Heart Disease

Valvular heart disease is a significant health problem and contributes to more than 44,000 deaths in United States annually.\textsuperscript{5} The causes of valvular heart disease include congenital malformations such as bicuspid aortic valves and rheumatic disease which is secondary to bacterial infection. Congenital valve disease affects about 2% of the general
population. More specifically, bicuspid aortic valve disease is the most common congenital heart defect. As the name suggests, the bicuspid aortic valve is typically made of 2 unequal-sized leaflets rather than the normal three. Bicuspid aortic valve disease is often asymptomatic during childhood. In adulthood, complications commonly arise and the burden of disease from bicuspid aortic valve disease is more significant than any other congenital heart disease. Despite this heavy economic burden, our understanding of bicuspid aortic valve disease is limited. Incidence of valvular heart disease dramatically increases to more than 13.3% over the age of 75. The disease may manifest as valvular stenosis, an obstruction of outflow, or as regurgitation, a defective closure resulting in backward flow. Congenital valve malformations tend to cluster in families among both close and distant relatives, suggesting a genetic component to this disease. However, underlying molecular mechanisms responsible for congenital valve disease are still not fully understood.

1.3 Hypoplastic Coronary Artery Disease

Congenital coronary anomalies affect up to 5% of the general population. Most of them are asymptomatic and therefore often unrecognized. However, people with major congenital coronary artery malformation are at greater risk for myocardial ischemia, infarction and sudden cardiac death. Hypoplastic coronary artery disease (HCAD) is a rare congenital coronary abnormality. It is characterized by malformation of one or more major branches of the coronary arteries with a marked decrease in luminal diameter and length. HCAD can be asymptomatic, but it can be associated with myocardial infarction and sudden cardiac death under stress, usually in the form of intense physical activity. However, the molecular mechanisms responsible for development of HCAD remain unclear.
1.4 **Formation of the Four-Chambered Heart**

Heart development is a complex process involving many coordinated processes including, migration, differentiation and proliferation of cardiac progenitors. The cardiac progenitors are derived from three distinct origins: the anterior lateral plate mesoderm or the primary heart field; the second heart field which is located dorsal and anterior to the primary heart field; and the cardiac neural crest from the dorsal neural tube.\textsuperscript{22-24} In addition, the epicardium is also a source of cardiac progenitors which give rise to coronary arteries and possibly cardiomyocytes.\textsuperscript{24, 25} Cardiac morphogenesis starts from the fusion of the 2 patches of anterior lateral plate mesoderm to form the cardiac crescent at embryonic day (E) 7.5 in the mouse embryo.\textsuperscript{26} By E8 the primitive heart (or heart tube) is formed. As the heart tube forms, cells from the second heart field migrate into the dorsal aspect to the heart tube in the pharyngeal mesoderm. Upon rightward looping of the heart tube, they cross the pharyngeal mesoderm and populate the outflow tract with contributions also from the cardiac neural crest. As the heart develops, the primitive heart undergoes specification processes including chamber specification, septation and trabeculation. The atrial and ventricular septum starts to form at E10. Multiple primordia contribute to a central mesenchymal mass, including the mesenchyme on the leading edge of the primary atrial septum, the atroventricular endocardial cushions, and the cap of mesenchyme on the spina vestibule.\textsuperscript{27, 28} Fusion of these components closes the ostium primum, completing atrial and atroventricular septation. The mitral and tricuspid valves are derived from the endocardial cushion while the aortic and pulmonary valves are formed from endocardial cushions and with a contribution of neural crest cells.\textsuperscript{29} The formation of atroventricular septum and valves is tightly regulated.
by coordinated cell proliferation, apoptosis and remodelling. By E14.5, a fully functional four-chambered heart is formed in mice.\textsuperscript{26}

1.5 Formation of the Coronary Arteries

The development of the coronary vessels is an important event at the later stages of cardiogenesis. Coronary development starts at E9.5 in mice from proepicardial organ (PEO) located at the pericardial surface of the septum transversum close to the sinus venosus.\textsuperscript{30, 31} Cells from PEO migrate and spread over the surface of the myocardium to become the epicardium. Concurrent with formation of the epithelium, some epithelial cells migrate further into the subepicardial space and undergo epithelial-mesenchymal transition (EMT) to become mesenchymal cells, which migrate throughout the developing myocardium and give rise to vascular smooth muscle cells, fibroblasts and possibly endothelial cells, and form coronary vasculature of the heart. The proximal ends of the coronary arteries connect to the ascending aorta through coronary orifices at the level of the left and right sinuses of the semilunar valves. Coronary veins connect to the right atrium via the coronary sinus. By E15.5, a complete coronary vascular system is established.\textsuperscript{30, 31} Over the past decade, many factors have been shown to be crucial in the formation of the coronary arteries,\textsuperscript{30, 32} but the role of NOS3 in this process is still not well understood.

1.6 Atrioventricular Valve Development

The atrioventricular (AV) valve development starts at E9 with the formation of endocardial cushions. A subpopulation of endocardial cells of the endocardial cushion undergoes endothelial-to-mesenchymal transition (EndMT) and provides the primary cell source for the development of AV valves.\textsuperscript{33} Proliferation of the mesenchymal cells and matrix deposition extend the cushions into the cardiac lumen and form primordia of each
distinct valve. This is followed by elongation and remodeling of the valve primordia, which leads to the gradual maturation of valves by E15. EndMT is a crucial process for proper formation of AV valves. Transforming growth factor (Tgfβ), bone morphogenetic protein (Bmp)-2, and Snail1 have been shown to promote EndMT and valve formation. In addition, Notch-dependent NOS3 activation has been shown to promote EndMT in the developing atrioventricular (AV) canal from E9 to E11.5 via activation of the PI3-kinase/Akt pathway.

1.7 Factors in Heart Development

The formation of the heart is highly regulated and complex in nature. Studies have shown both environmental and genetic factors play a crucial role in heart development. Well known environmental factors such as drug abuse, alcohol abuse and maternal diabetes all have been linked to as risk factors for congenital heart disease. In addition, several lines of evidence also support a genetic contribution to congenital heart disease. Over the past 50 years, through the use of genetically modified animals various factors have been identified to govern different aspects of heart development. In 2003, the completion of the human genome project allowed us to further test the importance of these factors in humans through the use of gene sequencing. These factors can be grouped into 2 categories: first, transcription factors that are responsible for initiating and regulating heart development and second, growth factors that acts as effectors to guide heart development.

1.7.1 Transcription Factors in Heart Development

Gata4 has been shown to be crucial in both human and murine heart development. Interestingly, Gata4 is one of the earliest transcription factors
expressed in the developing heart. More specifically, Gata4 mRNA can be detected as early as E7.0 in the pre-cardiac mesoderm and during formation and bending of the heart tube in endocardium, myocardium and pre-cardiac mesoderm at E8.0. It is worth noting that abundant Gata4 continues to be expressed in the heart throughout adulthood. Transgenic mice with inactivation of the Gata4 gene die during embryonic development due to the failure of ventral morphogenesis and heart tube formation between E8.0-E9.0. In addition, functional mutation of GATA4 in humans has been linked to congenital heart disease. Together, as a master regulator of cardiogenesis, Gata4 is required during embryonic heart development in both mice and humans.

Nkx2.5, a direct binding partner of Gata4 through its homeodomain, has also been shown to be involved in early heart development. As one of the earliest identified factors associated with human congenital heart disease, Nkx2.5 has been studied quite extensively throughout different organisms. Mutations have been identified in patients with a variety of congenital heart malformations including septal defects, conotruncal abnormalities, and atrioventricular conduction defects. Similar to Gata4, Nkx2.5 is also expressed early during heart development. Nkx2.5 is thought to act together with Gata4 to control the complex and precise regulation of heart development.

Heart development involves the precise looping and formation of the heart chambers, the development of the cardiac conduction system as well as the coronary vascular system. Wilm’s tumor -1 (Wt1) is a transcription factor that is involved in the formation of the coronary vasculature system. Wt1 has been shown to regulates epicardial epithelial to mesenchymal transition (EMT), a process which is required for coronary artery formation. Wt1 regulates epicardial EMT and heart development.
through multiple signaling pathways. It has been shown Wt1 promotes canonical Wnt/β-catenin signaling and Raldh2, a regulator of retinoic acid signaling.\textsuperscript{55, 56} By controlling epicardial EMT, Wt1 directs the coronary vasculature development in the heart.

Snail1, a zinc finger transcriptional repressor, has been studied extensively in developmental EMT. Mice with conditional knockout of \textit{Snail1} form a mesodermal layer with distinctly epithelial characteristics and die early in gastrulation around E7.5.\textsuperscript{57} Snail1 is also required for EMT-mediated mesoderm formation in zebrafish and for neural crest cell migration in mice.\textsuperscript{58} EMT is a crucial process during heart development which contributes to coronary artery formation, valve formation and septation of the ventricles and atria.\textsuperscript{59, 60} Taken together, the role of Snail1 in EMT and the importance of EMT in heart development, Snail1 dictates the proper formation of the heart by regulating EMT.

The transcription factors listed above are factors investigated in the current thesis, but this only represents the tip of the iceberg, as the majority of the transcription factors that are important during various aspects of heart development are beyond the scope of this thesis. Other transcription factors that are notable include Tbx5, Mef2C, NFATc1 and Pitx2. Tbx5 is a T-box containing transcription factor, that is expressed early in heart development at E8.0.\textsuperscript{61} \textit{TBX5} deficiency in humans results in Holt-Oram syndrome, a condition inherited as autosomal dominant, and the range of cardiac abnormalities include atrioventricular blocks, failure of septa formation and hypoplastic left heart syndrome, aortic stenosis, mitral valve prolapse, and the tetralogy of Fallot.\textsuperscript{62} Mef2C is a member of the myocyte-specific enhancer factor 2 (Mef2) family of transcription factors and is also important for cardiac muscle formation.\textsuperscript{63} In addition, recent discoveries reveal the specific role of Mef2C in second heart field development. Mef2C drives the
development of the right ventricle, septum formation and contributes to the proper
development of the atria, the outflow tract, pulmonary and aortic valves and endothelial
cells in the coronary artery.\textsuperscript{64} Specific deletion of \textit{Mef2C} in the heart results in right
ventricle defects as well failure of the heart tube to undergo looping morphogenesis.\textsuperscript{65}
NFATc1, a transcription factor required for heart valve formation in mice, starts to be
expressed early during murine heart development around E8.5.\textsuperscript{66, 67} Specific deletion of
\textit{NFATc1} in the hearts results in complete absence of pulmonary and aortic valves, leading
to congestive heart failure and death of the animal between E13.5-E17.5.\textsuperscript{67} Finally, Pitx2
is a crucial transcription factor in early heart development as it is responsible for the
establishment of left-right symmetry.\textsuperscript{68} \textit{PITX2} mutations in humans result in varies
complex syndromes such as Axenfeld-Rieger syndrome (ARS), iridogoniodygenesis
syndrome (IGDS), and sporadic cases of anterior segment mesenchymal dysgenesis.\textsuperscript{69-71}

1.7.2 Growth Factors in Heart Development

Growth factor signalling is a crucial component in heart development. It provides
the local niche for the migration and expansion of cardiomyocytes and other precursors in
the heart. Many growth factors have been identified, via mainly genetically modified
animals, to provide fundamental insights into their role in heart development. More
specifically, growth factors in the heart can acts as a chemokine that attracts precursors in
the epicardium to migrate into the myocardium and form coronary vasculature\textsuperscript{32} Without
the proper guidance provided by the local growth factors, coronary vasculature cannot be
correctly formed.

Vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF)
have been shown to the crucial in coronary artery formation. More specifically, inhibition
of VEGF and FGF decreases capillary formation and arterial growth.30, 32, 72 During early stages of coronary artery development, the expression FGF is high throughout the myocardium and VEGF expression is locally high at the site of early vascular tube formation.73, 74 It is worth noting that both factors have the potential to stimulate vascular growth in the embryonic heart.75 Myocardium specific overexpression of Fgf increases coronary artery density and branching, while Vegf conditional knockout impairs the recovery of coronary vessels from ischemic injury in mice.76, 77 Interestingly, the arterial diameter can be increased via the administration of anti-FGF-2 and anti-VEGF antibodies.78 These data suggest VEGF and FGF are crucial growth factors that is required during coronary artery formation.

Erythropoietin (EPO) is known for its vital role in the formation of red blood cells by binding to its receptor (EPOR).79 Through the use of genetically modified animal, Epo signalling through its receptor Epor was shown to be crucial in embryonic coronary artery formation. Epor is expressed in the epicardium and pericardium of the developing heart from E10 to E14. In Epor knockout mouse embryos, the epicardium was thin and detached from the myocardium. In addition, the coronary vessel network was severely affected with disorganized structures.79 This shows that inactivation of the Epo pathway leads to decreased epicardial precursors, and the detachment of the epicardium from the myocardium inhibited the crucial EMT process that is required for coronary artery formation.

1.8 Endothelial Activation and Thrombosis Formation

Endothelial activation generally refers to endothelial dysfunction or damage which may eventually contribute to vascular disease or thrombosis formation. Endothelial
activation represents a change in phenotype of the endothelial cells from the quiescent state towards a state that involves host defense response.\textsuperscript{80, 81} Most cardiovascular risk factors activate the endothelium which results in expression of chemokines, cytokines, and adhesion molecules that interact with the immune system to target inflammation to damaged tissues.\textsuperscript{80, 81} The molecular change involved in endothelial activation is switching from NO-mediated cellular processes, such as cGMP signalling or s-nitrosylation, to the activation of ROS signalling.\textsuperscript{82} This process is largely mediated by the uncoupling of NOS.\textsuperscript{83} Uncoupling of NOS3 is related to the depletion of important cofactor BH4, phosphorylation of specific residues of NOS3, mainly on residues serine 114, threonine 495, serine 615, serine 633 and serine 1177, increased S-glutathionylation and increased production of peroxynitrite.\textsuperscript{83, 84} NOS3 ROS production deteriorates cellular function via phosphorylation of transcription factors, induces adhesion molecule production and protease activation. Endothelial activation and ROS production can be a part of the normal host defense. It is when this process is extended, prolonged and/or in combination of the pro-inflammatory stimuli that results in endothelial damage.\textsuperscript{85} The damaged endothelium then contributes to vascular disease such as atherosclerosis or thrombosis formation.

Endothelium integrity not only depends on the extent of injury, but also on the ability of the endogenous capacity to repair.\textsuperscript{86} Repair mechanisms mainly occur in two ways. First is the replication of the mature endothelial cells which replaces the lost and damaged cells. Second is recruitment of circulating progenitor cells. These cells originate from the bone marrow and upon injury can be recruited to the site of injury to differentiate into endothelial cells.\textsuperscript{87, 88} Mobilization of these cells is in part NO-
dependent. Factors that have been shown to improve endothelial function and NO bioavailability such as statins and exercise have also been shown to promote endothelial progenitor cell mobilization.\textsuperscript{89-91} Therefore, it is a balance of repair and extent of endothelial damage that ultimately determines the integrity of the endothelium. Endothelial activation and damage, if unresolved, may result in thrombosis formation and vascular disease.

Arterial and venous thrombosis and thrombus embolization can be life threatening and if left untreated may lead to death. Arterial and venous thrombosis are blood clots that are formed in arteries and veins. The arterial clot is predominantly caused by platelets aggregation while a venous thrombus is mainly composed of fibrin and red blood cells and variable amount of platelets and white cells.\textsuperscript{92-94} Thrombi can occlude vessels locally at the site of formation or travel to other organs through blood and cause occlusion in distal vessels. Occlusions in the brain or the heart are especially dangerous and can cause stroke or heart attacks. However, thrombi may be spontaneously formed and lysed by local conversion of plasminogen to plasmin, a proteolytic enzyme which degrades plasma fibrinogen.\textsuperscript{95} The dynamics of platelet deposition and thrombus formation are modulated by the type of injury and the local geometry at the site of damage.\textsuperscript{92-94} Arterial thrombosis formation involves 3 distinct steps. First, platelets adhere at the recognition sites and are activated by the thromboactive substrates. The second step involves platelets spreading on the surface of attachment. Finally, platelets aggregate with each other to form a platelet plug. This process greatly depends on the efficiency of platelet recruitment and local geometry. Once platelets aggregate, other blood cells are recruited such as red blood cells, neutrophils and sometimes monocytes.\textsuperscript{92-}
The formation of a thrombus on the heart valve usually involves prosthetic valves. The process of thrombus formation is initiated during heart valve replacement surgery. The damaged peri-valvular tissue and prosthetic materials activate platelets as soon as blood starts flowing across the prosthetic valve. This results in the immediate deposition of platelets. In addition, the abnormal hemodynamic characteristics of mechanical prosthetic devices promote platelet activation. It is worth noting that bioprosthetic valves made from animals such as pig are considerably less thrombogenic mainly because of the biological properties of materials used in their construction. The spontaneous formation of a thrombus on the aortic valve is extremely rare in humans with only less than a dozen of case reports in the literature. However, aortic valve thrombosis is extremely dangerous, as it could lead to embolization, and subsequent cerebrovascular events such as stroke, myocardial infarction and death. Surgery is the only available treatment for these patients. Anti-coagulant therapy is still under debate as to whether the beneficial effects outweigh the deleterious effects. To our knowledge, spontaneous aortic valve thrombosis has never been studied in animal models.

### 1.9 Aortic Valve Calcification

Calcific aortic valve stenosis (CAVS) is the most common valvular disease. CAVS is characterized by progressive narrowing of the aortic valve resulting in aortic stenosis, calcification and thickening of the aortic valves and aortic valve regurgitation. Traditionally, calcified aortic valve stenosis has been associated with normal aging and valve degeneration. However, recent studies suggest that CAVS can be attributed to a genetic origin. Mutations in *Notch1*, an important factor that is involved in many developmental processes, has been shown to cause aortic valve disease similar to
CAVS.\textsuperscript{111} Notch1 functions by repressing the activities of Runx2, a central transcriptional regulator in osteoblast cell fate.\textsuperscript{111} The origin of calcification in the aortic valve is still under debate. Studies have shown that calcification originates from circulating progenitor cells that would reside on the aortic valve and differentiate into bone marrow–derived type I collagen\textsuperscript{+} osteocalcin (OCN)\textsuperscript{+} CD45\textsuperscript{+} subpopulation of mononuclear adherent cells, these cells then cause ossification.\textsuperscript{112,113} Another hypothesis is that valvular interstitial cells undergo differentiation and they would gain an osteoblast-like phenotype and cause calcification.\textsuperscript{114,115} It is likely a contribution of both scenarios that result in valve calcification. Environmental influences may also play a role in CAVS. Cardiovascular risk factors such as smoking, hypercholesterolemia, hypertriglyceridemia, diabetes mellitus, and chronic kidney disease all have been shown to contribute to CAVS.\textsuperscript{116} Currently, the only clinical treatment for CAVS is valve replacement surgery. Effective medical treatments to alter the course of CAVS are non-existent.

1.10 Nitric Oxide Synthase

Nitric oxide (NO) is produced from the guanidino group of L-arginine in an NADPH-dependent reaction catalyzed by a family of NO synthase (NOS) enzymes in living organisms.\textsuperscript{117,118} Originally identified as a vasodilatory agent, NO is now recognized as an important signalling molecule involved in a wide range of physiological and pathophysiological processes including cell growth, apoptosis, antithrombosis, neurotransmission, and immunological regulation.\textsuperscript{118,119} The wide range of functions and physiological importance of NO were recognized by the Nobel committee and awarded the 1998 Nobel prize of medicine to Drs. Furchgott, Ignarro and Murad for their contributions towards the discovery of NO. The vasodilatory function of NO was first discovered by Dr.
Furchgott and was termed endothelium-derived relaxing factor (EDRF). \textsuperscript{120} Later on, EDRF was identified as NO by Dr. Ignarro. \textsuperscript{121} The ultimate signalling of NO through cGMP was shown by Dr. Murad. \textsuperscript{122} There are 3 distinct isoforms of NOS: neuronal NOS (NOS1), inducible NOS (NOS2) and NOS3. Each NOS isoform is encoded by a distinct gene and is expressed in a variety of tissues and cell types. NO produced from NOS1 and NOS3 is involved in intracellular and intercellular signalling, whereas the high output NO produced by NOS2 is associated with inflammatory processes. \textsuperscript{118, 119} NO produced by different isoforms of NOS contributes towards signalling in the neuronal, immune, reproductive, vascular and most importantly, cardiovascular systems. \textsuperscript{118, 119} Shear stress induces NOS3 expression in the cardiovascular system during chick embryonic development. \textsuperscript{123} In addition to shear stress, NOS3 can also be induced and activated by Krüppel-like factor 2 (Klf2), an endothelial transcription factor that is crucially involved in vasculogenesis. \textsuperscript{124, 125} In the adult heart, NO released from NOS3 has several major roles including coronary vasodilation and tonic inhibition of mitochondrial O\textsubscript{2} consumption. \textsuperscript{118, 126} NO may also play a role in the muscarinic-cholinergic inhibition of β-adrenergic-stimulated chronotropy, \textsuperscript{127} inotropy, \textsuperscript{126} and atrioventricular nodal conduction. The effects of NO are mediated by the cGMP-dependent signaling and protein modification through S-nitrosylation. \textsuperscript{118, 128} In cGMP-dependent signalling, NO would activate downstream guanylate cyclase which leads to the production of cGMP. Binding of cGMP to the regulator domain of protein kinase G or cGMP dependent protein kinase induces a conformation change which stops the inhibition of catalytic core and allows phosphorylation of other downstream proteins. Protein kinase G is a serine/threonine-specific protein kinase that phosphorylates a number of important biological targets. \textsuperscript{118, 119} These targets drive many cellular processes which may regulate
 smooth muscle relaxation, platelet function, sperm metabolism, cell division and nucleic acid synthesis. Other NO mediated signalling includes S-nitrosylation. This is defined by the process of covalent incorporation of NO into thiol groups forming S-nitrosoprotein (SNO). This modification allows activation or deactivation of proteins. The activation or deactivation of proteins depends on the type of protein. For example, caspases that are important in cellular apoptosis have been shown to be inhibited by SNO. On the other hand, metalloproteinases which are important in degrading extracellular proteins are activated by SNO. SNO is an extremely complex process which remains poorly understood.

1.11 NOS3 Expression in the Embryonic Heart

NOS3 is expressed in the heart early during mammalian embryonic development. Although this expression is not restricted in the heart, other organs such as lung, liver, gastrointestinal tract, reproductive organs and brain hippocampus all have NOS3 expression. Immunohistochemical analysis in mice using NOS3 specific antibodies revealed that the heart, including cardiomyocytes, start to express NOS3 at E9.5 and expression remains high up to E13.5. Starting from E14.5, levels of NOS3 expression decrease in both atria and ventricles. After E19.5 low NOS3 levels are still detectable and this level of NOS3 expression in the myocardium remains to adulthood. Parallel with NOS expression, there are also changes in guanylate cyclase and cGMP production indicating the NO-cGMP signaling pathway in the heart development. The production of NO from NOS3 regulates the voltage-dependent L-type Ca\textsuperscript{2+} current in cardiomyocytes during early embryonic development and has been shown to promote cardiomyogenesis from mouse embryonic stem cells. It is important to note that although NOS1 and
NOS2 are expressed during cardiogenesis, NOS1 and NOS2 knockout mice have normal cardiac phenotype.

1.12 NOS3 and Heart Development

Septal defects are the most common cardiac malformations in humans. However, the molecular mechanisms that govern the formation of atrial and ventricular septum remain poorly understood. In recent years, through the use of genetic knockout mice, several transcriptional factors including Gata4, Nkx2.5 and Tbx5 have been identified to be critical in the development of atrial and ventricular septum. In addition, mutations in these factors are associated with congenital septal defects in humans. Feng et al. show that deficiency in NOS3 results in congenital septal defects and heart failure, and is accompanied by 85% postnatal mortality. All mortalities occur within the first 7 days after birth. Post-mortem examination show NOS3−/− mice have a high incidence of congenital septal defects with 64% atrial septal defects and 11% ventricular septal defects. Congenital septal defects can be a result of improper fusion of the atroventricular cushions during embryonic heart development. In addition, increases in apoptosis in atroventricular cushions can also contribute to congenital septal defects. In order to investigate the role of NOS3 in septal development, myocardial apoptosis was analysed in WT and NOS3−/− hearts at E12.5, a crucial time point in which the atroventricular cushions start to fuse together. Feng et al. show that there is an overall increase in the apoptotic activities in the NOS3−/− compared with WT hearts as measured by both caspase 3 activity and cytosolic DNA fragments. In order to determine the apoptotic activity in the atroventricular cushion, TUNEL staining was used. Feng and colleagues show that there is a significant increase in the apoptotic activity in the region of the atroventricular cushions, which may contribute at least in part to the increased
incidence of congenital septal defects seen in the postnatal day 1 NOS3\textsuperscript{-/-} mice.\textsuperscript{136} Low levels of NO produced by NOS3 inhibit apoptosis via S-nitrosylation of caspase-3, a critical protease responsible for the execution of apoptosis, and by promoting expressions of cytoprotective proteins including heme oxygenase-1, heat shock protein 70 (HSP70) and metallothionein.\textsuperscript{119} In addition, NO has also been shown to inhibit mitochondrial cytochrome c release and decrease permeability transition pore opening via mitochondrial membrane depolarization and inhibition of Ca\textsuperscript{2+} accumulation.\textsuperscript{139} These mechanisms may explain the increased apoptosis in the embryonic heart of NOS3\textsuperscript{-/-} mice.

Many factors may regulate the expression of NOS3 during embryonic heart development. Bio-informatics analysis of the mouse NOS3 locus showed three canonical Tbx5 binding sites, two of which are flanked by Gata binding site that binds to both Tbx5 and Gata4 with high affinities.\textsuperscript{140} Using genetically modified animals, Nadeau \textit{et al.} showed that endocardial specific knock-down of Tbx5 results in atrial septal defects with a 100% penetrance, suggesting Tbx5, a potential upstream regulator of NOS3, is also crucial in the proper formation of the atrial septum.\textsuperscript{140} Furthermore, compound haploinsufficiency of Tbx5 and NOS3 exacerbates the cardiac phenotype caused by deletion of a single Tbx5 allele from endocardial cells, suggesting that NOS3 may be a genetic modifier of Tbx5.\textsuperscript{140} Interestingly, decreases in enzyme activity and NO production in an 894G>T polymorphism of the NOS3 gene are associated with increased risks of CHD.\textsuperscript{141-143} Environmental factors and maternal conditions including psychological stress, hypertension and diabetes have been linked to increased risks of congenital heart disease.\textsuperscript{13} It has been shown that these environmental and maternal conditions can decrease NOS3 expression and/or activity.\textsuperscript{144, 145} The finding that NOS3, an enzyme that may be regulated by environmental conditions,
interacts with Tbx5, suggests the importance of gene–environment interactions in the setting of congenital heart disease and may help to explain the variable expressivity of the same mutation among affected family members and the complex inheritance patterns of congenital heart disease.\textsuperscript{140, 146}

In addition to the congenital septal defect, cardiac hypertrophy is also seen in \textit{NOS3}\textsuperscript{−/−} mice at postnatal day 0.\textsuperscript{136} From gross inspection and quantification, the \textit{NOS3}\textsuperscript{−/−} hearts are significantly larger than WT hearts. In order to determine if the cardiac function is impaired, \textit{in vivo} heart shortening was determined in the anesthetized P1 mice using ultrasound crystals that were placed on the surface of the heart. Feng \textit{et al.} show that percent shortening is significantly decreased in \textit{NOS3}\textsuperscript{−/−} compared to WT hearts. In addition, LV chamber size is significantly increased in \textit{NOS3}\textsuperscript{−/−} hearts. Furthermore, severe pulmonary congestion with focal alveolar edema is also seen in \textit{NOS3}\textsuperscript{−/−} mice at P1. Cardiac dysfunction and pulmonary congestion are typical clinical manifestations of heart failure. These results suggest that the higher mortality in postnatal \textit{NOS3}\textsuperscript{−/−} mice is likely due to the development of heart failure after birth.\textsuperscript{136} However, the exact cause of heart failure remains unclear. Congenital heart defects and decreased capillary density in the heart and lungs may contribute to the development of heart failure after birth in \textit{NOS3}\textsuperscript{−/−} mice.\textsuperscript{147-150}

\subsection{1.13 NOS3 and Its Upstream Activators}

In addition to transcription factors, NOS3 activity and expression are also regulated by a serine/threonine kinase Akt, also known as protein kinase B (PKB).\textsuperscript{151-153} Akt is an important mediator of phosphatidylinositol-3 kinase (PI3K) signaling that regulates a wide variety of cellular functions including survival, growth, proliferation, glucose uptake, metabolism, and angiogenesis.\textsuperscript{154} The effects are achieved through the
regulation of genes and proteins involved in these processes including NOS3.\textsuperscript{155,156} There are three Akt isoforms, Akt1, Akt2 and Akt3, which have similar structures and molecular sizes (57 kDa). All three Akt isoforms are expressed in the embryo starting from two-cell stage to virtually every organ during embryonic development. Inhibition of PI3K or Akt attenuates cardiomyocyte differentiation in embryonic stem cells, suggesting a critical role of PI3K/Akt signaling in early stage cardiomyocyte differentiation.\textsuperscript{157} Three Akt isoforms have similar but yet distinct physiological roles. Targeted disruption of the \textit{Akt1} gene in mice results in growth retardation with 20\% reduction in body size as well as septal defects.\textsuperscript{158,159} Akt2 knockout mice display insulin resistance and growth retardation while Akt3 knockouts show reduced brain size.\textsuperscript{160,161} It is worth noting that the septal defects seen in the \textit{Akt1}\textsuperscript{-/-} resembles to that of the \textit{NOS3}\textsuperscript{-/-} mice.\textsuperscript{136,158} Akt1\textsuperscript{-/-} mice showed perimembranous and muscular ventricular septal defects as well as atrial septal defects which are similar to those of \textit{NOS3}\textsuperscript{-/-} mice.

The effect of Akt is mediated in part by upregulation of \textit{Gata4} expression via phosphorylation of Gsk-3\(\beta\).\textsuperscript{162} Gsk3\(\beta\) inhibits the DNA binding activity of Gata4. Upon phosphorylation by Akt, Gsk3\(\beta\) exits the nucleus, which curtails the inhibitory effect of Gsk3\(\beta\) on \textit{Gata4}, leading to increases in Gata4 activation.\textsuperscript{162} Gata4 is a transcriptional factor that belongs to an evolutionarily conserved family of zinc finger-containing proteins, which has 6 members.\textsuperscript{163} Gata-1, -2 and -3 are expressed in hematopoietic stem cells while Gata-4, -5 and -6 are expressed in various mesoderm- and endoderm-derived tissues including the heart, liver, lung, gonad, and gut. Gata4 is a critical regulator of early cardiogenesis. Homozygous \textit{Gata4} knockout mice die at E9.5 and display defects in heart and foregut morphogenesis.\textsuperscript{45} Using tetraploid embryo complementation, Zhao \textit{et al.} (2008)
showed that Gata4 controls cardiomyocyte differentiation in mice. It appears that in the
Gata4
-/- mice, initiation of cardiomyocyte differentiation starts normally, but progenitors
cannot differentiate into terminal cardiomyocytes as shown by the lack of smooth muscle
actin, myosin heavy chain or sarcomeric actin staining. In humans, heritable mutations of
GATA4 result in congenital heart defects including, atrial septal defects, ventricular septal
defects, atrioventricular septal defects, pulmonary valve thickening or insufficiency of
cardiac valves. Interestingly, the promoter region of NOS3 contains Gata4 binding
sites. Studies have shown that Gata4 binds to these sites and increases NOS3 promoter
activity, suggesting Gata4 also serves as an upstream activator of NOS3.

1.14 NOS3 and Its Downstream Effectors

A classic pathway by which NO mediates its biological function is through
cGMP-dependent protein kinase G (PKG) signaling. Activation of PKG has been
shown to up-regulate varies downstream transcription factors that are important in heart
development. Thus, a deficiency in NOS3 decreases NO and cGMP production, and
may lead to decreased PKG activation and down-regulation of transcription factors in
heart development such as Gata4. Gata4 is a master regulator of heart development, and
controls the expression of many growth factors including VEGF, FGF and EPO, that are
important for coronary vessel formation and heart formation. NO can exert its
biological effect through other mechanisms. First, NO can cause oxidation of iron-
containing proteins such as ribonucleotide reductase. Ribonucleotide reductase is an
important enzyme in formation of formation of deoxyribonucleotides from
ribonucleotides. In addition, NO can activate proteins via sulphydryl group
nitrosylation. Nitrosylation involves the covalent binding of a nitric oxide into thiol
groups, to form S-nitrosothiol (SNO). S-nitrosylation has since been shown to be ubiquitous in biology.\textsuperscript{173-175} Dysfunction in S-nitrosylation has been associated with cardiovascular disease, Parkinson's disease, Alzheimer's disease and Amyotrophic Lateral Sclerosis (ALS). Finally, there is an emerging role of S-nitrosylation in cancer biology.\textsuperscript{176-179}

### 1.15 NOS3 Promotes Cardiomyocyte Proliferation

NOS3 is important to cardiomyocyte proliferation during early postnatal cardiac development.\textsuperscript{180, 181} To assess proliferation, cardiomyocytes were isolated from neonatal mice born within 24 hours and cultured for up to 96 hours. Cell proliferation was determined by bromodeoxyuridine (BrdU) incorporation and cell counts. Lepic \textit{et al.} showed that cultured NOS3\textsuperscript{−/−} cardiomyocytes displayed fewer cells and lower BrdU incorporation compared with WT cardiomyocytes.\textsuperscript{181} In order to analyze if the decreased cardiomyocyte proliferation is related to the level of NO produced by NOS3, NOS3\textsuperscript{−/−} and WT cardiomyocytes were treated with an NO donor and a NOS inhibitor, respectively. Treatment with the NO donor diethylenetriamine NONOate increased BrdU incorporation and cell counts in NOS3\textsuperscript{−/−} cardiomyocytes. Inhibition of nitric oxide synthase activity using N\textsuperscript{G}-nitro-L-arginine methyl ester (L-NAME) decreased the level of BrdU incorporation and cell counts in WT cardiomyocytes. Furthermore, to investigate cardiomyocyte proliferation \textit{in vivo}, neonatal mice were treated with BrdU. Deficiency in NOS3 significantly decreased BrdU labeling indexes in neonatal hearts.\textsuperscript{181} These data suggest that NO production from NOS3 is necessary for postnatal cardiomyocyte proliferation, providing a crucial role of NOS3 during postnatal heart development. However, despite decreases in cell proliferation, the overall size of the heart is enlarged.
in NOS3−/− as compared to WT mice (Figure 1A). This is probably due to the fact that cardiomyocyte proliferation is low and cardiac hypertrophy is the predominant response during early postnatal heart development.182, 183

1.16 NOS3 Promotes Postnatal Heart Maturation

The fetal and neonatal hearts develop through both hyperplasia and hypertrophy, which are increases in cell number and cell mass, respectively.183, 184 During early postnatal cardiac development, proliferation still occurs, albeit at a much lower level compared to fetal hearts. However, shortly after birth cardiomyocytes undergo binucleation, accompanied by a cessation of proliferation and a complete switch to hypertrophic growth.182, 183 In rodents this transition from hyperplastic growth to hypertrophic growth occurs within approximately 1-2 weeks after birth.182, 183 Embryonic heart development is characterized by the expression of cardiac specific proteins such as atrial natriuretic peptide (ANP) and proteins that form the contractile apparatus such as myosin heavy chain (MHC). After birth, as the heart gradually gains a mature phenotype, ANP expression is decreased and there is also a switch from beta to alpha isoform of MHC in cardiomyocytes.185 In the mature myocardium, α-MHC is the predominant isoform.186, 187 Lepic et al confirmed these postnatal changes in the WT mice and showed that cardiac ANP expression is progressively decreased from postnatal day 1 to day 7 while the expression of α-MHC is increased.181 On the other hand, in the NOS3−/− hearts, the levels of ANP remain high and the levels of α-MHC are significantly decreased at postnatal day 7, suggesting that NOS3 deficiency disturbs the normal temporal changes of ANP and α-MHC during postnatal heart development. These data support the notion that NOS3 plays an important role in promoting postnatal heart maturation.181 However, the
underlying molecular mechanisms responsible for these changes are not fully understood. Studies have shown that NF-κB, a transcription factor that is involved in inflammation, cell survival and growth, can modulate the expression of ANP and α-MHC.188,189 In addition, NO can increase or decrease the expression of NF-κB depending on its concentrations and the cell types.190,191 It is possible that NO produced from NOS3 may act through the NF-κB pathway to modulate expression of ANP and α-MHC in postnatal hearts.

1.17 NOS3 and Myocardial Capillary Development

NOS3 is crucial in the formation of capillaries and angiogenesis.149 To investigate the role of NOS3 in capillary development, myocardial capillary densities were measured morphometrically in P1 mouse hearts. Zhao et al. showed that myocardial capillary densities were decreased in $NOS3^{-/-}$ compared with WT mice.149 Next, in vitro tube formation of cardiac endothelial cells was investigated on Matrigel. Lack of NOS3 impaired tube formation of endothelial cells. In order to analyze if the decreased tube formation is related to the level of NO produced by NOS3, $NOS3^{-/-}$ and WT endothelial cells were treated with an NO donor and a NOS inhibitor, respectively. In vitro tube formation was inhibited by N$^{G}$-nitro-L-arginine methyl ester (L-NAME, a NOS inhibitor) in wild-type cells and restored by a NO donor, diethylenetriamine-NO, in $NOS3^{-/-}$ cells. In addition, angiogenesis was evaluated via implanting Matrigel in the myocardium of the adult heart. Following 3 days of implantation, significant angiogenesis is seen in the implanted Matrigel in WT mice. The angiogenic response was significantly decreased in the $NOS3^{-/-}$ mice. These data show that deficiency in NOS3 impairs myocardial
angiogenesis and capillary development, suggesting an important role of NOS3 in myocardial capillary development.149

1.18 NOS3 and Aortic Valve Development

The morphogenesis of the heart valves occurs concomitantly with changes in the cardiac morphology and in a complex process that includes initiation, cushion formation, elongation, valve remodeling and maturation.7, 34 Valve development is initiated during cardiac looping when the primary myocardium secretes a hyaluronan-rich matrix called cardiac jelly that projects into the lumen at the atrioventricular junction and the outflow tract at E9.0 in mice. The underlying myocardial cells produced factors including bone morphogenetic protein-2 (BMP2), BMP4 and transforming growth factor (TGF)-β, which activate the overlaying endocardium. At E10.5, the activated endocardial cells undergo EMT to become spindle shaped migratory cells (mesenchymal phenotype) and invade the cardiac jelly. Proliferation of the mesenchymal cells and matrix deposition extend the cushions into the cardiac lumen and form primordium of each distinct valve. This is then followed by elongation and remodeling/thinning of the valve primordial at E12.5, which leads to the gradual maturation of the valves that are rich in elastin, fibrillar collagen and proteoglycans. During valve remodeling, cell proliferation decreases, and subsequently there is little to no proliferation of valve interstitial cells in the adult. Formation of four distinct valves is a result of septation of the outflow tract into aorta and pulmonary trunk giving rise to aortic and pulmonary valves, and fusion of the atrioventricular canal endocardial cushions giving rise to mitral and tricuspid valves.29

Normal aortic valves have 3 distinct cusps (or leaflets) while bicuspid aortic valves have only 2 cusps.6 Lee et al. were the first to show that deficiency in NOS3
results in the formation of bicuspid aortic valves. The adult NOS3\(-/-\) mice had high incidences (5/12, 42%) of bicuspid aortic valves.\(^{192}\) Subsequent studies confirmed bicuspid aortic valves with an incidence of about 30% in NOS3\(-/-\) mice.\(^{193}\) In patients with bicuspid aortic valves, the common subtypes are fusion of right and left coronary leaflets (R-L) as well as fusion of right and non-coronary leaflets (R-N).\(^{6,194}\) In NOS3\(-/-\) mice, the bicuspid aortic valve is the R-N subtype in which the left leaflet develops normally.\(^{193}\) Data from the Feng lab also showed that NOS3\(-/-\) mice have bicuspid aortic valves.\(^{195}\) Furthermore, these mice have significant aortic regurgitation at postnatal day 1 as demonstrated by pulsed-wave Doppler and color flow Doppler echocardiography. The data is consistent with aortic regurgitation seen in patients with bicuspid aortic valves.\(^{196}\)

Notch signalling is essential for early embryonic development of aortic valves and postnatal repression of calcium deposition in the aortic valves. Mutations in the NOTCH1 gene cause autosomal-dominant anomalies of aortic valves and severe valve calcification in humans.\(^{111}\) In mice, Notch1 transcripts are the most abundant in the developing aortic valves. Furthermore, Notch1 inhibits the activity of Runx2, a central transcriptional regulator of osteoblast cell fate. Thus, decreases in Notch1 signaling result in developmental defect in the aortic valve and postnatal calcification that causes progressive aortic valve disease.\(^{111}\) Additionally, Notch signalling is also required for the EMT process in endocardial cushions during early valve development.\(^{197}\) Interestingly, recent studies have shown that Notch signalling activates NOS3 and its downstream target, soluble guanylyl cyclase via PI3K/Akt pathway.\(^{37}\) Therefore, NOS3 is likely a potential downstream target of Notch signalling that governs aortic valve development and postnatal remodeling.
1.19 NOS3 and Thrombosis

The role of NO in thrombosis formation has been investigated in animal models using NOS inhibitors and its substrate L-arginine. Stagliano et al. have shown infusion of L-NAME, a NOS inhibitor, caused an increase in platelet adhesion in a rat brain stroke model.\textsuperscript{198} In a glomerular thrombosis model, administration of endotoxin increased NO production and this effect was inhibited by infusion of L-NAME. Kidneys from rats given endotoxin and L-NAME showed enhanced thrombosis in glomeruli as compared with control with either L-NAME or endotoxin treated alone.\textsuperscript{199} In a canine model of coronary occlusion, L-arginine treatment increased lysis of thrombus in the coronary arteries and inhibited platelet aggregation \textit{ex vivo}.\textsuperscript{200} The importance of NOS3 in the prevention of thrombosis formation has also been demonstrated in the \textit{NOS3} knockout mice.\textsuperscript{201} These mice showed increased susceptibility to thrombosis, stroke and atherosclerosis.\textsuperscript{202-205} Exogenously, treatment with a NO-donor inhibited the upregulation of platelet surface glycoprotein including selectins and integrins, which are important for platelet aggregation.\textsuperscript{206} The molecular basis of the NO effect on platelets has been mainly attributed to cGMP dependent signalling.\textsuperscript{207-209} The increased production of cGMP leads to protein kinase G stimulation which results in fibrinogen binding to calcium-dependent heterodimeric complex of glycoproteins.\textsuperscript{207-209} This glycoprotein complex serves as an activation-dependent receptor for adhesive proteins such as fibrinogen.\textsuperscript{210} Binding of fibrinogen to glycoproteins mediated platelet aggregation which is crucial in thrombus formation. Decreased NO levels are also associated with thrombosis in human diseases.\textsuperscript{211, 212} It is well known that thrombosis is a common cause of myocardial infarction and unstable angina, and platelets from these patients show increased surface
expression of selectins and active glycoproteins. Interestingly, platelets isolated from these patients produce less NO compared to patients with stable coronary artery disease. The role of human NOS3 polymorphisms in thrombosis has recently been studied. It has been shown that NOS3 polymorphism is associated with lower platelet NO production, higher superoxide release and an increased risk of myocardial infarction, hypertension, stroke, and stenosis of arteries. Taken together, these results suggest that NOS3 variants may influence thrombotic susceptibility in humans.

1.20 NOS3 and Aortic Valve Calcification

Aortic valve calcification can occur spontaneously with increasing age, atherosclerosis and disease conditions such as diabetes and renal disease. It has been shown that NO inhibits calcification of vascular smooth muscle cells and differentiation of VSMCs into osteoblastic cells via inhibition of transforming growth factor-β (TGF-β)-induced phosphorylation of Smad2/3 and expression of TGF-β-induced genes such as plasminogen activator inhibitor-1. An inhibitor of cGMP dependent protein kinase increased osteoblastic differentiation and calcification of vascular smooth muscle cells. In addition, atorvastatin, a statin drug, inhibits calcification and enhances NO production in the hypercholesterolaemic aortic valves. NOS3 protein concentrations were increased in the atorvastatin treated group. Serum nitrite concentrations were decreased in the hypercholesterolaemic animals and increased in the group treated with atorvastatin. These data suggest that NOS3 may act through TGF-β to prevent calcification of vascular smooth muscle cells and the degree of calcification may relate to the amount of NO produced. Although the precise role of NOS3 on aortic valve calcification is not well understood, Bosse et al. have suggested that NOS3 signalling can
regulate Notch1, a key molecule in aortic valve calcification and genetically linked to human calcific aortic valve disease. They have shown that calcification of aortic valve interstitial cells can be prevented or accelerated by gain or loss of NO, respectively. In addition, overexpression of Notch1 can slow down the progression of valvular calcification induced by NO inhibition. These results suggest that NOS3 do play an important protective role against aortic valve calcification.

1.21 Summary of Heart Defects in NOS3−/− mice

Cardiac NOS3 expression starts early in cardiogenesis at E9.5 and remains high until E13.5. This also coincides with the crucial period of rapid heart development and formation of coronary arteries and aortic valves. Deficiency in NOS3 results in cardiac hypertrophy, congenital septal defects, and postnatal heart failure. In addition, myocardial capillary densities are decreased in NOS3−/− mice. Furthermore, deficiency in NOS3 results in high incidence of bicuspid aortic valves. These data suggest that NOS3 plays a critical role in embryonic heart development and morphogenesis of aortic valves and possibly coronary arteries (Figure 1.1). Although how lack of NOS3 leads to these cardiac phenotypes is not completely understood, possible mechanisms may include increases in apoptosis, decreases in cell proliferation, changes in gene expression, and decreases in EPDC migration and EMT. We anticipate that studies on these mechanisms of NOS3 during embryonic heart development may lead to therapeutic strategies in the prevention and treatment of congenital heart disease.
Figure 1.1. NOS3 is crucial during heart development. NO produced from NOS3 may promote coronary artery development, proper septal formation, proper valve formation, capillary development and cardiomyocyte maturation.
1.22 Rationale and Hypotheses

Overall Hypothesis:

Deficiency of NOS3 results in hypoplastic coronary arteries, atrioventricular valve insufficiency, and aortic valve calcification and thrombosis.

Overall Objectives (Figure 1.2):

1. Investigate the role of NOS3 in fetal coronary artery development.
2. Investigate the role of NOS3 in fetal atrioventricular valve development.
3. Investigate the role of NOS3 in maintaining normal aortic valve structure and function in adulthood and aging.

Study 1. Role of NOS3 in coronary artery development

Rationale:

NOS3 is expressed in the embryonic heart at E9.5. Studies have shown that NO production from NOS3 is critical in cardiogenesis. Previous studies have demonstrated that NOS3 plays an important role in embryonic heart development. Furthermore, NOS3 promotes cardiomyocyte proliferation and protects cardiomyocytes from apoptosis. Deficiency in NOS3 resulted in embryonic and postnatal abnormalities in heart development. NO from NOS3 is an important mediator of vasculogenesis and angiogenesis. Additionally, studies have shown that development of myocardial capillaries is impaired in NOS3 knockout mice. Coronary artery development is a complex process, which involves the migration of PEO to the looping heart, and subsequent migration and differentiation of epicardium-derived mesenchymal cells. The
molecular mechanisms regulating coronary artery development are still not well understood.

**Hypothesis:**
Deficiency in NOS3 impairs coronary artery development.

**Objectives:**
1. To analyze coronary artery development in $NOS3^{+/+}$, $NOS3^{-/-}$, $NOS3\text{Tg};NOS3^{-/-}$ and $NOS3\text{Tg}$ mice at E15.5 and P0.
2. To identify the cause of death in the $NOS3^{-/-}$ neonates.
3. To understand the signaling mechanisms by which NOS3 controls coronary artery development.

**Study 2. Role of NOS3 in atrioventricular valve development**

**Rationale:**

NOS3 is important for embryonic heart development. Deficiency in NOS3 leads to congenital septal defect, bicuspid aortic valves and coronary artery malformation.\textsuperscript{136, 192, 225} The temporal expression pattern of NOS3 peaks during atrioventricular valve formation, suggesting that NOS3 may participate in the development of the atrioventricular valves. However, the role of NOS3 in atrioventricular valve formation and endothelial to mesenchymal transition is not known.

**Hypothesis:**
Deficiency in NOS3 impairs atrioventricular valve formation and endothelial to mesenchymal transition.
Objectives:

1. To assess atrioventricular valves in $NOS3^{+/+}$, $NOS3^{-/-}$, $NOS3Tg;NOS3^{-/-}$ and $NOS3Tg$ mice at postnatal day 0.

2. To determine if atrioventricular valve defects in $NOS3^{-/-}$ mice result in functional deficits.

3. To understand the role of NOS3 in the formation of atrioventricular valves.

Study 3. Lack of NOS3 results in thrombosis, calcification and dysfunction of aortic valve

Rationale:

The spontaneous formation of a thrombus on the aortic valve is extremely rare in humans; there are only less than a dozen of case reports in the literature. However, aortic valve thrombosis is extremely dangerous, as it could lead to embolization, and subsequent cerebrovascular events such as stroke, myocardial infarction and death. These patients are limited to surgery as it is the only available and effective treatment. The cause of aortic valve thrombosis remains unclear. Mutations in Notch1, an important factor that is involved in many developmental processes, have been shown to cause aortic valve disease similar to calcified aortic valve stenosis. Additional evidence showed that NOS3 acts upstream of Notch1 and regulate its expression. This would suggest that disruption in NOS3 signalling would also result in phenotypes similar to that of Notch1 mutation. Studies have suggested that endothelial damage in bicuspid aortic valve or calcific aortic valve disease could potential result in the formation aortic valve thrombosis. To our knowledge, spontaneous aortic valve thrombosis has never
been studied in an animal model. Study 3 investigated the role of NOS3 in normal maintenance of aortic valve function and its deficiency results in aortic valve thrombosis, dysfunction and calcification in adulthood and aging.

Hypothesis:
Deficiency in NOS3 leads to aortic valve thrombosis, dysfunction and calcification in aging mice.

Objectives:
1. To investigate the role of NOS3 in the formation of aortic valve thrombosis in aging mice.
2. To assess aortic valve function in vivo in $NOS3^{+/+}$ and $NOS3^{-/-}$ mice at 1, 3, 5, 9 and 12 Months.
3. To analyze the degree of calcification and fibrosis of aortic valve leaflets in $NOS3^{+/+}$ and $NOS3^{-/-}$ mice.
4. To understand the role of NOS3 in aortic valve calcification.
Figure 1.2 Summary of studies 1, 2 and 3 on the role of NOS3 in embryonic heart development and adult aortic valve function.
1.23 References

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Nitric oxide synthase-3 deficiency results in hypoplastic coronary arteries and postnatal myocardial infarction

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“Nitric oxide synthase-3 deficiency results in hypoplastic coronary arteries and postnatal myocardial infarction.”
2.1 CHAPTER SUMMARY

Aims: Hypoplastic coronary artery disease is a rare congenital abnormality that is associated with sudden cardiac death. However, molecular mechanisms responsible for this disease are not clear. The aim of the present study was to assess the role of nitric oxide synthase-3 (NOS3) in the pathogenesis of hypoplastic coronary arteries.

Methods and Results: Wild-type (WT), NOS3<sup>−/−</sup> and a novel cardiac specific NOS3 overexpression mouse model were employed. Deficiency in NOS3 resulted in coronary artery hypoplasia in fetal mice and spontaneous myocardial infarction in postnatal hearts. Coronary artery diameters, vessel density and volume were significantly decreased in NOS3<sup>−/−</sup> mice at postnatal day 0. In addition, NOS3<sup>−/−</sup> mice showed a significant increase in ventricular wall thickness, myocardial volume and cardiomyocyte cell size compared with WT mice. Lack of NOS3 also down-regulated the expression of Gata4, Wilms tumor-1, vascular endothelial growth factor, basic fibroblast growth factor and erythropoietin, and inhibited migration of epicardial cells. These abnormalities and hypoplastic coronary arteries in the NOS3<sup>−/−</sup> mice were completely rescued by the cardiac-specific overexpression of NOS3.

Conclusion: NOS3 is required for coronary artery development and deficiency in NOS3 leads to hypoplastic coronary arteries.
2.2 INTRODUCTION

Congenital coronary anomalies affect up to 5% of the general population.\textsuperscript{1,2} Most of these do not have clinical signs or symptoms, and are often unrecognized. However, severe congenital coronary artery malformation is associated with myocardial ischemia, infarction and sudden cardiac death.\textsuperscript{3} Hypoplastic coronary artery disease (HCAD), a rare congenital coronary abnormality, is defined by malformation of one or more major branches of the coronary arteries with a marked decrease in luminal diameter and length.\textsuperscript{4-6} HCAD can be asymptomatic, but is often associated with myocardial infarction and sudden cardiac death under stress, such as during intense physical activity.\textsuperscript{3,7} However, the molecular mechanisms responsible for embryonic development of HCAD are still unknown.

Nitric oxide (NO) is produced from the guanidino group of L-arginine in an NADPH-dependent reaction catalyzed by a family of NO synthase (NOS) enzymes.\textsuperscript{8} Originally identified as a vasodilatory agent, NO is now recognized as an important signaling molecule involved in a wide range of physiological processes including apoptosis, angiogenesis and cell growth.\textsuperscript{8,9} There are 3 distinct isoforms of NOS: neuronal NOS (nNOS, NOS1), inducible NOS (iNOS, NOS2) and endothelial NOS (eNOS, NOS3). Interestingly, NOS3 is expressed in cardiomyocytes during early mammalian embryonic heart development.\textsuperscript{10} Immunohistochemical analysis revealed that the heart starts to express NOS3 at E9.5 and the expression remains high until E13.5. Starting from E14.5 the levels of NOS3 expression decreases in both atria and ventricles.\textsuperscript{10} The expression of NOS3 in the heart peaks during coronary artery development,\textsuperscript{10,11} suggesting its potential importance. However, the role of NOS3 in embryonic coronary artery development is still not clear.
The signaling milieu within the developing heart is critical to coronary vasculogenesis.\textsuperscript{11} Transcription factors such as \textit{Gata4} and Wilms tumor-1 (Wt1),\textsuperscript{12, 13} as well as growth factors including vascular endothelial growth factor (VEGF), basic fibroblast growth factor (FGF) and erythropoietin (EPO)\textsuperscript{14, 15} are essential in the formation of coronary arteries.\textsuperscript{11, 15} We have previously shown a key role of NOS3 to capillary vessel development in the heart.\textsuperscript{16} In the present study, we hypothesized that NO production from NOS3 within the embryonic heart is a critical signaling molecule in the development of coronary arteries and deficiency in \textit{NOS3} results in hypoplastic coronary arteries.

2.3 METHODS

2.3.1 Animals

Breeding pairs of \textit{NOS3} \textsuperscript{-/-} (stock No. 002684) and wild-type C57BL/6 (\textit{NOS3} \textsuperscript{+/+}) mice were purchased from Jackson Laboratory (Bar Harbor, Maine). A breeding program was carried out to produce neonates. Genotyping of \textit{NOS3} \textsuperscript{-/-} and \textit{NOS3} \textsuperscript{+/+} mice was performed by a polymerase chain reaction (PCR) method using genomic DNA prepared from tail biopsies. A timed breeding program was carried out. Vaginal plug was monitored in the morning of each day and a plug was considered 0.5 days into pregnancy. Embryonic hearts were collected at E12.5, E15.5 and postnatal day 0 (P0). All procedures involving mouse husbandry and manipulation were in accordance with the guidelines of the Canadian Council of Animal Care and approved by the Animal Use Subcommittee at the University of Western Ontario, Canada.
2.3.2 Generation of Human NOS3 Transgenic Mice

A new line of cardiac-specific mice overexpressing human isoforms of \textit{NOS3} (\textit{NOS3}\textsuperscript{Tg}) was generated. Briefly, human NOS3 complementary DNA was inserted into the \(\beta\)-myosin heavy chain promoter expression vector to permit the expression of human NOS3 only during embryonic development and specifically in the heart. Genotypes were identified by PCR using genomic DNA from tail biopsies. Primer sequence is shown in Table 1. Relative protein expression of varies genotypes were done via western blotting and immunohistochemical staining.
Table 2.1 Gene name, accession number and primers used.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession #</th>
<th>Primer Sequence</th>
</tr>
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<tbody>
<tr>
<td>Hu-NOS3</td>
<td>NM_000603.3</td>
<td>Sense: 5’ CTC GTG TGA AGA ACT GGG AGG T 3’&lt;br&gt;Antisense: 5’ GTG GCA TAC TTG ATG TGG TTG 3’</td>
</tr>
<tr>
<td>28S</td>
<td>NR_003279.1</td>
<td>Sense: 5’ TTG AAA ATC CGG GGG AGA G 3’&lt;br&gt;Antisense: 5’ ACA TTG TTC CAA CAT GCC AG 3’</td>
</tr>
<tr>
<td>Gata4</td>
<td>NM_008092.3</td>
<td>Sense: 5’ CAC TAT GGG CAC AGC AGC TC 3’&lt;br&gt;Antisense: 5’ GCC TGC GAT GTC TGA GTG AC 3’</td>
</tr>
<tr>
<td>VEGFa</td>
<td>NM_001025250.2</td>
<td>Sense: 5’ GAT TGA GAC CCT GGT GGA CAT C 3’&lt;br&gt;Antisense: 5’ TCT CCT ATG TGC TGG CTT TGG T 3’</td>
</tr>
<tr>
<td>FGF</td>
<td>NM_008006.1</td>
<td>Sense: 5’ CAA GGG AGT GTG TGC CAA CC 3’&lt;br&gt;Antisense: 5’ TGC CCA GTT CGT TTC AGT GC 3’</td>
</tr>
<tr>
<td>EPO</td>
<td>NM_007942.2</td>
<td>Sense 5’ GGA ATT GAT GTC GCC TCC AG 3’&lt;br&gt;Antisense: 5’ GCA GCA GCA TGT CAC CTG TC 3’</td>
</tr>
<tr>
<td>Wt1</td>
<td>NM_144783.2</td>
<td>Sense: 5’ GAT GTG CGG CGT GTA TCT GG 3’&lt;br&gt;Antisense: 5’ GCT GGT CTG AGC GAG AAA ACC T 3’</td>
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2.3.3 ECG Monitoring

Short-term monitoring of ECG limb lead was performed in anesthetized mice with needle electrodes inserted subcutaneously. ECG was recorded with Powerlab Chart 5.0 (AD Instruments, Colorado Springs, CO).
2.3.4 TTC Staining

Neonatal hearts were first dissected out of the body, cut in half and washed in PBS to remove blood. Next, they were incubated in 1% TTC solution at room temperature for 1 hour. Finally, images are taken on a microscope for macro samples (Discovery V8., Zeiss, Germany).

2.3.5 Three-Dimensional Reconstructions and Histological Analysis

Three-dimensional (3D) reconstructions of the heart and coronary arteries were performed using α-smooth muscle actin stained serially sections of P0 and E15.5 hearts through the AMIRA® software (Template Graphics Software, USA). Briefly, stained serially sections were imaged (Observer D1, Zeiss, Germany). These images were then used for 3D reconstructions of the heart and measurements of coronary artery diameter, abundance and myocardial wall thickness. All measurements were made at similar anatomical location to ensure consistency. Calculations of myocardial and coronary artery volume were made using the AMIRA® program.

2.3.6 Ex vivo Heart Cultures

E12.5 NOS3+/+ and NOS3−/− hearts were explanted and cultured in M199 medium (Sigma-Aldrich, USA). Hearts were stabilized for 2 hours prior to any treatment. Both NOS3+/+ and NOS3−/− hearts were beating throughout the entirety of the experiment. NOS3+/+ and NOS3−/− hearts were treated with 100 µM ODQ (Sigma-Aldrich Chemie, USA) and 2 mM 8-Bromo-cGMP (Sigma-Aldrich Chemie, USA), respectively for 6 hours. Hearts were then collected and mRNA levels of Wt1 and Gata4 were analyzed by real time RT-PCR.
2.3.7 Co-culture of EPDCs and Cardiomyocytes

E13 eGFP\(^+\) hearts were planted on a 35 mm petri dish for 3 days to allow outgrowth of epicardial cells, which are epicardial-derived cells (EPDCs). After 3 days, the hearts were removed and eGFP\(^+\) EPDCs were cultured for 3 more days prior to co-culture with \(NOS3^{+/+}\) or \(NOS3^{-/-}\) cardiomyocytes. \(NOS3^{+/+}\) and \(NOS3^{-/-}\) cardiomyocytes were isolated according to previous protocol with the following modifications. E13 \(NOS3^{+/+}\) and \(NOS3^{-/-}\) embryos were collected and hearts were carefully dissected out of the embryo. The co-culture system functions similar to a wound healing assay system (Fig. 2.8A). Cardiomyocytes isolated from E13 \(NOS3^{+/+}\) and \(NOS3^{-/-}\) mice were plated on the inside of the barrier overnight with or without the treatment of adenoviral NOS3 or lacZ. eGFP\(^+\) EPDCs were cultured on the outside of the barrier for 2 hours before the barrier was removed. After the barrier was removed, the eGFP\(^+\) cells were given 72 hours to migrate. Images were then taken under a confocal microscope (LSM 510 Meta, Zeiss, Germany). In order to obtain a complete view, a tiled scan was performed to piece together 16 images at 40x into a unified image.

2.3.8 General Tissue Processing

Neonatal \(NOS3^{+/+}\), \(NOS3^{+/-}\), \(NOS3^{-/-}\), \(NOS3^{Tg/-}\)/\(NOS3^{-/-}\) and \(NOS3^{Tg}\) mice were used for analysis. Neonatal hearts and lungs were isolated and fixed in 4% paraformaldehyde, dehydrated, and embedded in paraffin. Five micro-meter transverse serial sections were mounted onto albumin/glycerin coated glass slides.

2.3.9 Real Time RT-PCR

Total RNA was isolated from cultured fetal hearts with a RNA isolation kit (Qiagen, Canada). cDNA was synthesized using M-MLV (Invitrogen, Canada) reverse
transcriptase and random primers (Invitrogen, Canada). Real-time PCR was conducted using SYBR Green PCR Master Mix as per manufacturer's instructions (Eurogentec, USA). The oligonucleotide primers used in this study are summarized in Supplementary Table 1. Samples were amplified for 35 cycles using MJ Research Opticon Real-Time PCR machine. The expression levels of Gata4, VEGFa, FGF, EPO, and Wt1 in relation to 28S rRNA as a loading control were obtained.

2.3.10 Immunohistochemistry and Immunofluorescence

After deparaffination and rehydration of the slides, microwave antigen retrieval was applied by heating them for 10 min at 98°C in a citric acid buffer (0.01 M in aqua-dest, pH 6.0). Inhibition of endogenous peroxidase was performed with a solution of 0.3% H2O2 in phosphate buffered saline (PBS) for 30 min. The slides were incubated overnight with either 1:500 anti-troponin I (Santa Cruz Biotechnology, USA), 1:3,000 anti-α-smooth muscle actin (Sigma- Aldrich, USA), 1:500, 1:300 anti-Wt1 (Calbiochem, USA) or 1:1000 anti-NOS3 (BD Transduction Laboratories, USA). Next, the secondary antibodies was added for 60 min with either 1:200 horse anti-mouse biotin (Vector Laboratories, USA) or 1:200 goat anti-rabbit biotin (Vector Laboratories, USA) in PBS. Subsequently, slides were incubated with ABC reagent (Vector Laboratories, USA) for 30 min. For visualization, the slides were incubated with 400 μg/ml 3-3’di-aminobenzidin tetrahydrochloride (Sigma-Aldrich Chemie, USA) and 100 μL of 30% H2O2 dissolved in PBS for 10-15 min. Counterstaining was performed with 0.1% hematoxylin (Merck, Germany). For immunofluorescence staining, following primary antibody incubation, 1:500 goat anti-rabbit fluorescent secondary antibody (Jackson ImmunoResearch, USA) was used. Nuclei were stained with 1:50000 Hoechst 33342
Cardiomyocyte membranes were stained with 1:300 WGA (Vector Laboratories, USA).

2.3.11 Western Blot Analysis

Forty micrograms of protein lysates were subjected to separation on a 12% SDS-PAGE gel, followed by electrotransfer to nitrocellulose membranes. Blots were probed with specific antibodies against NOS3 (BD Biosciences, USA) and GAPDH (Santa Cruz Biotechnology, USA), respectively. Signals were detected by the chemiluminescence detection method.

2.3.12 Heart Function Measurements

Left ventricular (LV) ejection fraction and fractional shortening were measured using Vevo 2100 (Visual Sonics, Canada). Briefly, 2-dimensional images of the heart were obtained in short-axis view using a dynamically focused 40 MHz probe. The M-mode cursor was positioned perpendicular to the LV anterior and posterior walls. The LV internal end-diastolic dimension (LVIDd) and LV internal systolic dimension (LVIDs) were measured from M-mode recordings. LV ejection fraction was calculated as: EF (%) = [(LVIDd)^3 - (LVIDs)^3]/(LVIDd)^3×100. Fractional shortening was calculated as: FS (%) = (LVIDd-LVIDs)/LVIDd×100. The M-mode measurements of the left ventricular ejection fraction and fractional shortening were averaged from 3 cycles.

2.3.13 EPDC Outgrowth

E12.5 NOS3^{+/+} and NOS3^{--} hearts were planted on a 35 mm petri dish for 3 days to allow outgrowth of epicardial cells, which are epicardial-derived cells (EPDCs). After
3 days, these hearts were imaged under phase contrast (Observer D1, Zeiss, Germany). Their maximum migration distance was measured.

2.3.14 Statistical Analysis

Data are presented as mean±SEM. Survival curves were created by the method of Kaplan and Meier and compared by log-rank test. Unpaired Student’s t test was used for 2 group comparisons. One way ANOVA followed by Student-Newman-Keuls test was performed for multi-group comparisons. Two way ANOVA test was performed for multi-group, multi-treatment comparisons. A two-sided $P<0.05$ was considered statistically significant. All statistical analyses were performed using GraphPad Prism software 3.0.

2.4 RESULTS

2.4.1 Characterization of $\text{NOS3}^{\text{TG}}$ and $\text{NOS3}^{\text{TG}};\text{NOS3}^{\text{-/-}}$ mice.

In order to study the specific role of NOS3 in coronary artery development, a cardiac-specific NOS3 transgenic mouse ($\text{NOS3}^{\text{TG}}$) was generated under the control of $\beta$-myosin heavy chain promoter. Expression of human NOS3 was detected only during embryonic development and specifically in the heart of $\text{NOS3}^{\text{TG}}$ mice (Figure 2.1A-D). The $\text{NOS3}^{\text{TG}}$ mice were then crossed with $\text{NOS3}^{\text{-/-}}$ to create the $\text{NOS3}^{\text{TG}};\text{NOS3}^{\text{-/-}}$ mouse, an animal that lacks NOS3 in all organs except the heart during embryogenesis (Figure 2.1B, D). Since the gaseous molecule NO can freely diffuse between cells, NOS3 overexpression in cardiomyocytes rescued NOS3 expression in $\text{NOS3}^{\text{-/-}}$ hearts.
**Figure 2.1** Generation of mice with cardiomyocyte specific overexpression of human nitric oxide synthase-3 (hu-NOS3) during embryonic development. (A) Construct used for the generation of mice with cardiomyocyte specific overexpression of hu-NOS3 under the control of β-myosin heavy chain (MHC). (B) NOS3 protein levels in the heart at E12.5, P0 and 5 months of age by Western blot analysis. Tg;−/− and Tg;+/+ indicate NOS3<sub>Tg</sub>;NOS3<sup>−/−</sup> and NOS3<sub>Tg</sub>, respectively. (C) Expression of the hu-NOS3 transgene was restricted in the heart at P0. Hu-NOS3 mRNA was analyzed by RT-PCR. (D) Western blot analysis showed decreased NOS3 expression from NOS3<sup>+/+</sup> to NOS3<sup>+/−</sup> to NOS3<sup>−/−</sup> animals. (E) NOS3 immunostaining at E12.5 showing expression of the Hu-NOS3 transgene. Endogenous NOS3 expression was mostly located in the endocardium and capillary endothelium in the NOS3<sup>+/+</sup> mice. NOS3<sup>+/−</sup> mice showed no detectable expression of NOS3. NOS3<sub>Tg</sub>;NOS3<sup>−/−</sup> mice showed strong NOS3 expression in the myocardium, but not in the endocardium or capillary endothelium. NOS3<sub>Tg</sub> mice showed strong NOS3 expression in the myocardium (transgene), endocardium and capillary endothelium (endogenous). Scale bar = 30 μm.
2.4.2 Deficiency in NOS3 results in spontaneous myocardial infarction.

Deficiency in NOS3 resulted in 73% mortality within the first 4 days after birth (Figure 2.2E). Coronary artery malformation may lead to myocardial ischemia and cell death, resulting in myocardial infarction. To assess if myocardial infarction was the cause of death, P0 hearts were subjected to triphenyltetrazolium chloride (TTC), troponin I and hematoxylin/eosin (H/E) staining. TTC staining showed that NOS3<sup>−/−</sup> mice had a large area of tissue death near the apex of the heart (Figure 2.2A). Troponin I is a part of the troponin complex that is integral to muscle contraction and decreased levels of troponin I represent cardiomyocyte death.<sup>18</sup> Our data showed troponin I immunostaining was decreased in the area of infarct in NOS3<sup>−/−</sup> mice (Figure 2.2A). H/E staining showed typical wavy fibers near the border of the infarct area with disappearance of nuclei and intense eosinophilic cytoplasm (Figure 2.2A), which are signs of acute myocardial infarction.<sup>19</sup> ECG monitoring showed ST elevation and QRS inversion in neonatal NOS3<sup>−/−</sup> mice, indicating myocardial ischemia and possible cardiac hypertrophy, respectively (Figure 2.2A). Spontaneous myocardial infarction was seen in 8/15 (53%) NOS3<sup>−/−</sup> animals at P0 (Figure 2.2B). Consequently, left ventricular ejection fraction and fractional shortening were significantly decreased in the NOS3<sup>−/−</sup> compared to NOS3<sup>+/+</sup> mice (<i>P</i>&lt;0.05, Figure 2.2C-D). These abnormalities in NOS3<sup>−/−</sup> mice including myocardial infarction, cardiac dysfunction, and postnatal mortality were all rescued by cardiac-specific overexpression of NOS3 (NOS3<sup>Tg</sup>,NOS3<sup>−/−</sup> mice, Figure 2.2A-E).
**Figure 2.2** Myocardial infarction and postnatal survival in NOS3<sup>−/−</sup> mice. (A) Evidence of spontaneous myocardial infarction in NOS3<sup>−/−</sup> mice at P0. TTC staining shows a large area of tissue death near the apex of the heart. The images are adjacent cross sections of the heart near the apex. Troponin I staining shows a significant loss of troponin I in the infarct myocardium. H/E staining shows waviness of fibers near the border of the myocardium with intense eosinophilic cytoplasm. ECG tracing shows significant ST elevation (Arrows) and QRS inversion (Arrowheads), representing signs of myocardial ischemia and possible cardiac hypertrophy, respectively. White bar: 1mm. Black bar: 40µm. (B) Spontaneous myocardial infarction in NOS3<sup>−/−</sup> animals at P0. * P<0.01 vs. NOS3<sup>+/+</sup>, NOS3<sup>Tg;−</sup>;NOS3<sup>−/−</sup> and NOS3<sup>Tg</sup> mice. Tg;<sup>−/−</sup> and Tg;<sup>+/+</sup> indicate NOS3<sup>Tg;NOS3<sup>−/−</sup></sup> and NOS3<sup>Tg</sup>, respectively. (C-D) Heart function assessed by echocardiography in NOS3<sup>+/+</sup>, NOS3<sup>−/−</sup>, NOS3<sup>Tg;NOS3<sup>−/−</sup></sup> and NOS3<sup>Tg</sup> mice at P0. N=5-6 mice per group. * P<0.01 vs. NOS3<sup>+/+</sup> mice, † P<0.01 vs. NOS3<sup>−/−</sup>. (E) Thirty-day survival after birth in NOS3<sup>+/+</sup>, NOS3<sup>−/−</sup>, NOS3<sup>Tg;NOS3<sup>−/−</sup></sup> and NOS3<sup>Tg</sup> mice. * P<0.001 vs. NOS3<sup>+/+</sup>, NOS3<sup>Tg;NOS3<sup>−/−</sup></sup> and NOS3<sup>Tg</sup> mice.
2.4.3 **Deficiency in NOS3 impairs coronary artery development.**

*NOS3<sup>−/−</sup>* and *NOS3<sup>+/−</sup>* hearts at P0 showed less branching and significant decreases in the left and right coronary artery diameters (Figure 2.3A-D). Three-dimensional reconstructions of the heart showed a significant decrease in coronary volume in *NOS3<sup>−/−</sup>* mice (*P*<0.01, Figure 2.3E, Figure 2.4A). Cardiomyocyte-specific NOS3 overexpression completely rescued these defects in the *NOS3<sup>−/−</sup>* mice (Figure 2.3A-E, Figure 2.4A).

Since a decrease in coronary artery volume may have been a result of a smaller myocardial mass, myocardial volume and thickness were analyzed. However, both myocardial volume and thickness were increased in *NOS3<sup>−/−</sup>* and *NOS3<sup>+/−</sup>* compared to *NOS3<sup>+/+</sup>* mice (*P*<0.01, Figure 2.4B-C). In order to determine if the increased myocardial volume and thickness are a result of cardiac hypertrophy, cardiomyocyte cell size and nuclear size of P0 hearts were measured. Cardiomyocyte cell and nuclear size was progressively increased in *NOS3<sup>−/−</sup>* and *NOS3<sup>+/−</sup>* compared to *NOS3<sup>+/+</sup>* mice (*P*<0.01, Figure 2.4D-E). Therefore, our data showed an increased myocardial mass accompanied by severe coronary artery hypoplasia. These defects *NOS3<sup>−/−</sup>* mice were completely rescued by cardiomyocyte-specific NOS3 overexpression (*P*<0.01, Figure 2.4B-E).

The coronary artery network is established by E15.5 in mice. To assess the onset of the coronary artery malformations, we further analyzed coronary artery development at E15.5. Coronary artery abundance, vessel diameter and volume were all significantly decreased while myocardial thickness was increased in *NOS3<sup>+/+</sup>* compared to *NOS3<sup>+/−</sup>* and *NOS3<sup>−/−</sup>* mice at E15.5 (*P*<0.01, Figure 2.5A-F). These results suggest that the onset of the defect is prior to E15.5. Consistent with our P0 data, these defects were completely rescued by cardiomyocyte-specific NOS3 overexpression (*P*<0.01, Figure 2.5A-F). Since
coronary artery formation at E15.5 was not impaired in $NOS3^{+/+}$ mice, all subsequent molecular analyses on early coronary artery development were carried out in $NOS3^{-/-}$ in comparison with $NOS3^{+/+}$ mice.
**Figure 2.3** Immunohistochemical analysis of coronary artery development at P0. (A) Heart sections were stained with vascular smooth muscle α-actin to identify coronary arteries. Coronary arteries were smaller and less abundant in NOS3<sup>−/−</sup> and NOS3<sup>+/−</sup> compared to NOS3<sup>++</sup> mice. Cardiac specific overexpression of NOS3 (NOS3<sup>Tg</sup>;NOS3<sup>−/−</sup>) restored coronary abundance in NOS3<sup>−/−</sup> mice. Coronary arteries are indicated by red arrows. Scale bar = 60 μm. (B-D) Left and right coronary artery diameter and abundance measured at 50 μm from the aortic orifice were significantly decreased NOS3<sup>−/−</sup> and NOS3<sup>+/−</sup> compared to NOS3<sup>++</sup> mice, which was restored in NOS3<sup>Tg</sup>;NOS3<sup>−/−</sup> mice. N=5 hearts per group. *P<0.01 vs. NOS3<sup>++</sup>; †P<0.01 vs. NOS3<sup>−/−</sup>. ‡P<0.01 vs. NOS3<sup>−/−</sup>. Tg;−/− and Tg;+/+ indicate NOS3<sup>Tg</sup>;NOS3<sup>−/−</sup> and NOS3<sup>Tg</sup>, respectively. (E) Coronary artery volume was significantly decreased NOS3<sup>−/−</sup> and NOS3<sup>+/−</sup> compared to NOS3<sup>++</sup> mice, which was restored in NOS3<sup>Tg</sup>;NOS3<sup>−/−</sup> mice. N=5 hearts per group. *P<0.01 vs. NOS3<sup>++</sup>; †P<0.01 vs. NOS3<sup>−/−</sup>.
Figure 2.4 3D reconstruction of coronary artery development at P0. (A) Coronary arteries shown in green were less abundant in \( NOS3^{-/-} \) and \( NOS3^{+/-} \) compared to \( NOS3^{+/+} \) mice. Cardiac specific overexpression of NOS3 restored coronary artery abundance in \( NOS3^{-/-} \) mice. Atria were excluded from the reconstruction in order to view the origins of the coronary arteries. (B-C) Ventricular wall thickness and myocardial volume were significantly increased in \( NOS3^{-/-} \) and \( NOS3^{+/-} \) compared to \( NOS3^{+/+} \) mice. Cardiac overexpression of NOS3 (\( NOS3^{Tg;NOS3^{-/-}} \)) restored myocardial thickness and volume to similar levels as in the \( NOS3^{+/+} \) mice. (D-E) Cross-section cardiomyocyte cell and nucleus size was significantly increased \( NOS3^{-/-} \) and \( NOS3^{+/-} \) compared to \( NOS3^{+/+} \) mice. Cardiac overexpression of NOS3 (\( NOS3^{Tg;NOS3^{-/-}} \)) restored myocardial thickness to similar levels as in the \( NOS3^{+/+} \) mice. N=4 hearts per group. \*\( P<0.01 \) vs. \( NOS3^{+/+} \) mice, \textasciitilde \( P<0.01 \) vs. \( NOS3^{+/-} \) mice, \$\( \ P<0.01 \) vs. \( NOS3^{-/-} \). Tg;/- and Tg;+/- indicate \( NOS3^{Tg;NOS3^{-/-}} \) and \( NOS3^{Tg} \), respectively.
**Figure 2.5** Coronary artery development at E15.5. (A) 3D reconstruction of E15.5 hearts. Coronary arteries shown in green were less abundant in *NOS3<sup>−/−</sup>* compared to *NOS3<sup>+/−</sup>* and *NOS3<sup>+/+</sup>* mice. Cardiac specific overexpression of NOS3 restored coronary artery abundance in *NOS3<sup>−/−</sup>* mice. Atria were excluded from the reconstruction in order to view the origins of the coronary arteries. (B-E) Coronary artery vessel diameter, abundance and volume were significantly decreased in *NOS3<sup>−/−</sup>* mice compared to *NOS3<sup>+/−</sup>* and *NOS3<sup>+/+</sup>* mice, which were restored in *NOS3<sup>Tg</sup>;NOS3<sup>−/−</sup>* mice. N=5 hearts per group. Tg;<sup>−/−</sup> and Tg;<sup>+/+</sup> indicate *NOS3<sup>Tg</sup>;NOS3<sup>−/−</sup>* and *NOS3<sup>Tg</sup>* respectively. (F) Ventricular wall thickness was significantly increased in *NOS3<sup>−/−</sup>* compared to *NOS3<sup>+/−</sup>* and *NOS3<sup>+/+</sup>* mice. Cardiomyocyte specific overexpression of NOS3 (*NOS3<sup>Tg</sup>;NOS3<sup>−/−</sup>*) restored myocardial thickness to similar levels as in the *NOS3<sup>+/+</sup>* mice. N=4 hearts per group. For B-F, *P<0.01 vs. *NOS3<sup>+/−</sup>* mice, † P<0.01 vs. *NOS3<sup>−/−</sup>*.
2.4.4 Down-regulation of transcription and growth factors in E12.5 NOS3−/− hearts.

Capillary networks in the embryonic heart begin to develop at E12.5, and are regulated by transcription and growth factors that are critical to heart morphogenesis and coronary artery formation.11 Our data showed that the mRNA levels of Gata4, VEGFa, FGF and EPO were significantly decreased in the NOS3−/− compared with NOS3+/+ hearts, and were completely restored in the NOS3Tg;NOS3−/− hearts (P<0.05, Figure 2.6A-D). Treatment with ODQ, a soluble guanylyl cyclase inhibitor significantly decreased Gata4 and Wt1 mRNA expression in the NOS3+/+ hearts (P<0.05, Figure 2.6E-F). Conversely, NOS3−/− hearts treated with 8-Bromo-cGMP, a cGMP analog showed a significant increase in Gata4 and Wt1 mRNA levels (P<0.05, Figure 2.6G-H). These data suggest that NOS3 regulates the expression of Gata4 and Wt1 through a cGMP-dependent signaling pathway.
Figure 2.6 Myocardial mRNA expression of transcription and growth factors. (A-D)

E12.5 hearts were collected and mRNA levels of *Gata4*, VEGFa, FGF and EPO were analyzed by real-time RT-PCR. The mRNA levels of *Gata4*, VEGFa, FGF and EPO were significantly decreased in *NOS3*<sup>-/-</sup> compared with *NOS3*<sup>+/+</sup> mice, which were restored by cardiac specific *NOS3* overexpression in the *NOS3*<sup>Tg;</sup>*NOS3*<sup>-/-</sup> hearts. N=6-7 per group. *P*<0.01 vs. *NOS3*<sup>+/+</sup> mice. †*P*<0.01 vs. *NOS3*<sup>-/-</sup> mice. Tg;/- and Tg;+/+ indicate *NOS3*<sup>Tg;</sup>*NOS3*<sup>-/-</sup> and *NOS3*<sup>Tg;</sup> respectively.

(E-H) E12.5 *ex vivo* heart cultures were used to investigate NOS3 signaling. Cultured *NOS3*<sup>+/+</sup> and *NOS3*<sup>-/-</sup> hearts were treated with 100 µM ODQ, a soluble guanylate cyclase inhibitor, or 2 mM 8-Br-cGMP, a cGMP analog, for 6 hrs. ODQ decreased *Gata4* and Wt1 mRNA levels in *NOS3*<sup>+/+</sup> hearts while 8-Br-cGMP restored *Gata4* and Wt1 mRNA levels in *NOS3*<sup>-/-</sup> hearts. N=3-4 heart cultures per group. *P*<0.05 vs. controls.
2.4.5 Deficiency in NOS3 decreases epicardium-derived cells (EPDCs) migration.

EPDCs express Wt1 and their migration into the myocardium is essential to the formation of coronary arteries.\textsuperscript{13,20} The number of Wt1\textsuperscript{+} epicardial cells was significantly decreased in \textit{NOS3}\textsuperscript{+/−} compared to \textit{NOS3}\textsuperscript{+/+} hearts at E12.5 (\textit{P}<0.01, Figure 2.7A-B). In addition, myocardial Wt1 mRNA levels were also significantly decreased in \textit{NOS3}\textsuperscript{+/−} compared with \textit{NOS3}\textsuperscript{+/+} mice (\textit{P}<0.01, Figure 2.7C). Decreased myocardial expression of Wt1 and number of Wt1\textsuperscript{+} epicardial cells were rescued in \textit{NOS3}\textsuperscript{Tg;NOS3}\textsuperscript{+/-} mice (\textit{P}<0.01, Figure 2.7A-C). To study the role of NOS3 in EPDCs migration, a co-culture system was employed (Figure 2.8A). EPDCs were isolated from E13 embryos overexpressing enhanced green fluorescence protein (eGFP). The cultured EPDCs were typical cobblestone shape and 100% positive for epicardin and Wt1. Cardiomyocytes was verified by α-actinin staining (Figure 2.8B). eGFP\textsuperscript{+} EPDCs were then co-cultured with either E13 \textit{NOS3}\textsuperscript{+/+} or \textit{NOS3}\textsuperscript{+/-} cardiomyocytes treated with adenoviral lacZ or adenoviral NOS3 for 72 hours. Our data showed migration of eGFP\textsuperscript{+} EPDCs was significantly decreased towards the \textit{NOS3}\textsuperscript{+/-} compared to the \textit{NOS3}\textsuperscript{+/+} cardiomyocytes (Figure 2.8C-D). However, EPDC migration was increased in cardiomyocytes treated with adenoviral NOS3 compared to adenoviral lacZ controls (Figure 2.8C-D). Finally, to assess EPDC migration and proliferation in the developing heart, \textit{ex vivo} heart explants were employed. Our data showed \textit{ex vivo} EPDC migration and proliferation were significantly decreased in the \textit{NOS3}\textsuperscript{+/-} compared with \textit{NOS3}\textsuperscript{+/+} hearts (\textit{P}<0.01, Figure 2.8E-F).
Figure 2.7 Characterization and quantification of Wt1⁺ epicardial progenitor cells. (A) E12.5 hearts were immunostained for Wt1 and representative images from NOS3⁺/⁺, NOS3⁺/-, NOS3Tg;NOS3⁻/- and NOS3Tg hearts are shown. The majority of Wt1⁺ cells are in the epicardium with limited expression in the myocardium. Scale bar = 60 μm. (B) Quantitative analysis showed a significant reduction in Wt1⁺ epicardial cells in NOS3⁺/- compared with NOS3⁺/⁺ hearts, which was restored by cardiac specific NOS3 overexpression in the NOS3Tg;NOS3⁻/- hearts. N=4-5. (C) Wt1 mRNA levels were analyzed in E12.5 hearts by real time RT-PCR. Wt1 mRNA expression was significantly decreased in the NOS3⁻/- compared with NOS3⁺/⁺ hearts, which was restored in NOS3Tg;NOS3⁻/- hearts. N=6-7 per group. For B and C, *P<0.01 vs. NOS3⁺/- mice, †P<0.01 vs. NOS3⁻/- mice, Tg;⁻/- and Tg;⁺/+ indicate NOS3Tg;NOS3⁻/- and NOS3Tg, respectively.
Figure 2.8 Migration of epicardium-derived cells (EPDCs). (A) EPDCs were isolated from E13 hearts and co-cultured with cardiomyocytes as shown. The number of EPDCs migrated towards the cardiomyocytes was determined. (B) A representative confocal tiled image of the EPDCs and cardiomyocyte co-culture. EPDCs (green) and cardiomyocytes were isolated from eGFP transgenic and C57BL6 wild-type mice, respectively. Cardiomyocytes were stained with cardiac α-actinin (red). (C) Quantitative analysis from (D) shows that EPDC migration was significantly decreased in NOS3−/− compared to NOS3+/+ cardiomyocyte co-cultures, which was restored by adenoviral NOS3 treatment. N=3-5 per group. *P<0.01 vs. NOS3+/+ mice. †P<0.01 vs. respective Ad-lacZ treated groups. (D) NOS3 expression in cardiomyocytes promoted EPDCs migration. Representative confocal tiled images of eGFP+ EPDCs co-cultured with NOS3+/+ and NOS3−/− cardiomyocytes, which were treated lacZ or NOS3 adenoviral constructs. (E) Representative images of EPDC outgrowth of E12.5 NOS3+/+ and NOS3−/− hearts. Scale bar = 250 μm. (F) Quantitative analysis shows that maximum EPDC migration distance was significantly decreased in NOS3−/− hearts compared with NOS3+/+ hearts. N=7-8, *P<0.01 vs. NOS3+/+ heart. (G) Proposed signaling pathway of NOS3 on coronary artery development. NOS3 promotes the expression of Gata4 and Wt1 via cGMP-dependent mechanisms. The production of growth factors (VEGFα, FGF and EPO) by Gata4 and EPDCs migration by Wt1 contribute to the critical role of NOS3 on coronary artery development. Dash lines represent signalling pathways shown by others.
2.5 DISCUSSION

Growing evidence suggests that NO plays an important role in embryonic heart development. To this end, we and others have previously shown that lack of NOS3 results in congenital septal defects and bicuspid aortic valves in mice.\(^{21-24}\) In addition, impaired capillary and microvessel development has been shown in the heart and lungs of \(NOS3^{-/-}\) mice, respectively.\(^{16, 25}\) The present study was carried out to further examine the role of NOS3 in coronary artery development. We demonstrated for the first time that deficiency of NOS3 leads to hypoplastic coronary arteries. These results show that NOS3 plays a critical role in normal coronary artery development (Figure 2.8G). To rescue the coronary phenotype in the \(NOS3^{-/-}\) mice, we generated a mouse with cardiomyocyte-specific overexpression of NOS3 during embryonic development. The \(NOS3^{Tg}\) mice had a normal cardiac and coronary artery phenotype. Importantly, cardiomyocyte-specific overexpression of NOS3 completely rescued the abnormal coronary artery development, cardiac hypertrophy and postnatal survival in the \(NOS3^{-/-}\) mice. The data strongly suggest that normal coronary artery development is driven by local NO signaling from endothelial cells and/or cardiomyocytes within the developing heart.

Our data showed that deficiency in NOS3 down-regulated the expression of \(Gata4\), VEGFa, FGF and EPO in the embryonic heart. Additionally, Wt1, a transcription factor critical for EMT and coronary artery formation, was also down-regulated and the number of EPDCs was decreased in the \(NOS3^{-/-}\) hearts. Furthermore, lack of NOS3 decreased EPDCs migration towards the cardiomyocytes. Flow dependent dilation and remodeling are important mechanisms in the regeneration of adult blood vessels.\(^{11, 15}\) However, initiation of embryonic coronary artery development relies more on \textit{de novo} arterial
plexuses formation that are independent of blood flow. Eventually at around E14.5, arterial plexuses are connected and the connection of coronary artery to the aorta is made. After this point, flow dependent dilation and remodeling in the coronary artery are important in establishing further expansion of the vessels similar to adult blood vessel generation.\textsuperscript{11, 15} This process, however, is not NOS3 dependent.\textsuperscript{26} Our results show that NOS3 promotes normal coronary artery development via increases in expression of transcription and growth factors, and EPDCs migration into the myocardium (see Figure 2.8G).

\textit{NOS3}\textsuperscript{−/−} mice have a high rate of postnatal mortality. We have previously shown that within 10 days after birth, mortality was 85\%, 38\% and 13\% for \textit{NOS3}\textsuperscript{−/−}, \textit{NOS3}\textsuperscript{+/−} and WT mice, respectively.\textsuperscript{21} Most animals died within the first 3 days after birth. Consistent with our finding, Han \textit{et al.} showed that 40\% of \textit{NOS3}\textsuperscript{−/−} offspring succumbed within the first hour of birth.\textsuperscript{25} However, they did not monitor their animal survival beyond one hour of birth. In the present study, the mortality of \textit{NOS3}\textsuperscript{−/−} mice was 73\% in the first 4 days after birth, which is in agreement with the previous studies.\textsuperscript{21, 25} Additionally, we demonstrated a dose-dependent cardiac dysfunction at P1 with the loss of one or both NOS3 alleles.\textsuperscript{21} Interestingly, this gene dose-dependent response was also observed in the coronary artery formation of \textit{NOS3}\textsuperscript{−/−}, \textit{NOS3}\textsuperscript{+/−} and \textit{NOS3}\textsuperscript{+/+} mice at P0 in the present study. These data suggest that hypoplastic coronary arteries may contribute at least in part to postnatal cardiac dysfunction and mortality in \textit{NOS3}\textsuperscript{−/−} mice.

Hypoplastic coronary artery disease (HCAD) is often associated with myocardial infarction and sudden cardiac death when the heart is stressed.\textsuperscript{7} In the present study, coronary artery malformation is accompanied by spontaneous myocardial infarction in
postnatal NOS3<sup>−/−</sup> mice. Our study demonstrated that NOS3 deficiency results in hypoplastic coronary arteries, a condition that mirrors HCAD in humans. Interestingly, recent studies have shown that a common 894G>T single nucleotide polymorphism, which reduces NOS3 activity, is associated with up to 2.3 fold increased risk of congenital heart disease, especially conotruncal heart defects. In addition, environmental factors and maternal conditions including psychological stress, hypertension and diabetes, which decrease NOS3 expression and/or activity, are associated with increased risks of congenital heart disease. Thus, it is possible that decreased NOS3 signaling may promote the development of congenital heart disease in patients with these environmental and maternal conditions. Although further studies are required to analyze NOS3 gene mutation in patients with HCAD, the present study suggests NOS3 is critical to coronary artery development and deficiency or mutation of NOS3 gene may lead to HCAD. Our study is the first to implicate NOS3 deficiency in the pathogenesis of HCAD and may help to design strategies in the diagnosis, prevention and treatment of HCAD in humans.
2.6 REFERENCES

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3 CHAPTER 3

Nitric Oxide Synthase-3 Promotes Embryonic Development of Atrioventricular Valves

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“Nitric Oxide Synthase-3 Promotes Embryonic Development of Atrioventricular Valves”
3.1 CHAPTER SUMMARY

Aims: Nitric oxide synthase-3 (NOS3) has recently been shown to promote endothelial-to-mesenchymal transition (EndMT) in the developing atrioventricular (AV) canal. The present study was aimed to investigate the role of NOS3 in embryonic development of AV valves. We hypothesized that NOS3 promotes embryonic development of AV valves via EndMT.

Methods and Results: To test this hypothesis, morphological and functional analysis of AV valves were performed in wild-type (WT) and NOS3\(^{-/-}\) mice at postnatal day 0. Our data show that the overall size and length of mitral and tricuspid valves were decreased in NOS3\(^{-/-}\) compared with WT mice. Echocardiographic assessment showed significant regurgitation of mitral and tricuspid valves during systole in NOS3\(^{-/-}\) mice. These phenotypes were all rescued by cardiac specific NOS3 overexpression. To assess EndMT, immunostaining of Snail1 was performed in the embryonic heart. Snail1 positive and total mesenchymal cells in the AV cushion were decreased in NOS3\(^{-/-}\) compared with WT mice at E10.5 and E12.5, which was completely restored by cardiac specific NOS3 overexpression. In cultured embryonic hearts, NOS3 promoted transforming growth factor (TGFβ), bone morphogenetic protein (BMP2) and Snail1 expression through cGMP. Furthermore, mesenchymal cell formation and migration from cultured AV cushion explants were decreased in the NOS3\(^{-/-}\) compared with WT mice.

Conclusion: NOS3 promotes AV valve formation during embryonic heart development and deficiency in NOS3 results in AV valve insufficiency.
3.2 INTRODUCTION

Valvular heart disease is a significant health problem and contributes to more than 44,000 deaths in United States annually.\textsuperscript{1} The causes of valvular heart disease include congenital malformation and rheumatic disease which is secondary to bacterial infection. Congenital valve disease affects about 2\% of the general population.\textsuperscript{2, 3} The disease may manifest as valvular stenosis, an obstruction of outflow, or as regurgitation, a defective closure resulting in backward flow. Congenital valve malformations tend to cluster in families among both close and distant relatives, suggesting a genetic component of this disease.\textsuperscript{4, 5} However, molecular mechanisms responsible for congenital valve disease are still not fully understood.

Nitric oxide synthase (NOS) enzymes convert L-arginine to L-citrulline and produce nitric oxide (NO), a signaling molecule involved in a wide range of physiological processes including apoptosis, angiogenesis, cell proliferation and differentiation.\textsuperscript{6-8} Three distinct isoforms of NOS has been identified: neuronal NOS (NOS1), inducible NOS (NOS2) and endothelial NOS (NOS3). The expression of NOS3 starts around E9.5 during early mouse embryonic heart development.\textsuperscript{9} This expression remains high until E13.5. By E14.5, the levels of NOS3 expression decrease in both atria and ventricles. After E19.5 low NOS3 levels are still detectable and this level of NOS3 expression in the myocardium remains into adulthood.\textsuperscript{9}

The atrioventricular (AV) valve development starts at E9 with the formation of endocardial cushions. A subpopulation of endocardial cells of the endocardial cushion undergoes endothelial-to-mesenchymal transition (EndMT) and provides the primary cell source for the development of AV valves.\textsuperscript{10} Proliferation of the mesenchymal cells and
matrix deposition extend the cushions into the cardiac lumen and form primordia of each distinct valve. This is followed by elongation and remodeling of the valve primordia, which leads to the gradual formation of valves by E15. The valves continue to grow and remodel throughout heart development and well after birth. EndMT is a crucial process for proper formation of AV valves. Transforming growth factor (TGFβ), bone morphogenetic protein (BMP)-2, and Snail1 have been shown to promote EndMT and valve formation. We have shown that NOS3 is important for embryonic heart development. Deficiency in NOS3 leads to congenital septal defect, bicuspid aortic valves and coronary artery malformation. In addition, Notch-dependent NOS3 activation has been shown to promote EndMT in the developing atrioventricular (AV) canal from E9 to E11.5 via activation of the PI3-kinase/Akt pathway. The temporal expression pattern of NOS3 peaks during AV valve formation along with its role in EndMT, suggesting that NOS3 may participate in the development of the AV valves. However, the role of NOS3 in the formation and functioning of AV valves has not been studied. In the present study, we hypothesized that NOS3 promotes embryonic development of AV valves via EndMT. To test this hypothesis, morphological changes and functional competence of AV valves were studied in wild-type (NOS3+/+), NOS3−/− and cardiac specific NOS3 overexpressing (NOS3Tg) mice. Furthermore, the role of NOS3 in endocardial EndMT and signaling pathway critical to AV valve development was also examined. Our study demonstrated that NOS3 promotes endocardial EndMT and embryonic AV valve development.
3.3 METHODS

3.3.1 Animals

Breeding pairs of NOS3<sup>−/−</sup> (stock No. 002684) and wild-type C57BL/6 (NOS3<sup>+/+</sup>) mice were purchased from Jackson Laboratory (Bar Harbor, Maine). A breeding program was carried out to produce neonates. Genotyping of NOS3<sup>−/−</sup> and NOS3<sup>+/+</sup> mice was performed by a polymerase chain reaction (PCR) method using genomic DNA prepared from tail biopsies. A timed breeding program was carried out. Vaginal plugging was monitored in the morning of each day and when plugged, was considered 0.5 days into pregnancy. Embryonic hearts were collected at E10.5, E12.5, and postnatal day 0. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication #85-23, revised 1996) and the experimental protocols were approved by Animal Use Subcommittee at Western University.

3.3.2 Generation of Human NOS3 Transgenic Mice

A new line of cardiac-specific mice overexpressing human isoforms of NOS3 (NOS3<sup>Tg</sup>) was generated. Briefly, human NOS3 complementary DNA was inserted into the β-myosin heavy chain promoter expression vector to permit the expression of human NOS3 only during embryonic development and specifically in the heart. Our previous studies have shown that the transgene is specifically expressed in the heart during embryonic development. Genotypes were identified by PCR using genomic DNA from tail biopsies.
3.3.3 General Tissue Processing

Neonatal \( NOS3^{+/+} \), \( NOS3^{-/-} \), \( NOS3^Tg/NOS3^{-/-} \) and \( NOS3^Tg \) mice were used for analysis. Hearts were isolated and fixed in 4% paraformaldehyde, dehydrated, and embedded in paraffin. Five micro-meter transverse serial sections were mounted onto albumin/glycerin coated glass slides.

3.3.4 Immunohistochemistry and Immunofluorescence

Established protocols were used with minor modifications \(^{18,20,23} \). Briefly, after deparaffination and rehydration of the slides, microwave antigen retrieval was applied by heating them for 10 min at 98°C in a citric acid buffer (0.01 M in aqua-dest, pH 6.0). Inhibition of endogenous peroxidase was performed with a solution of 0.3% \( \text{H}_2\text{O}_2 \) in phosphate buffered saline (PBS) for 30 min. The slides were incubated overnight with either 1:500 anti-Snail1 (Abcam, USA). Next, the goat anti-rabbit secondary antibody conjugated with biotin (1:200, Vector Laboratories, USA) was added for 60 min in PBS. Subsequently, slides were incubated with ABC reagent (Vector Laboratories, USA) for 30 min. For visualization, the slides were incubated with 400 \( \mu \text{g/ml} \) 3-3’di-aminobenzidin tetrahydrochloride (Sigma-Aldrich, USA) and 100 \( \mu \text{L} \) of 30% \( \text{H}_2\text{O}_2 \) dissolved in PBS for 10-15 min. Counterstaining was performed with 0.1% hematoxylin. The size and length of AV valves were measured at the proximal (hinge) and distal aspects of the leaflets as described previously. \(^{24} \)

3.3.5 Three-Dimensional Reconstructions and Histological Analysis

Three-dimensional (3D) reconstructions of the heart and AV valves were performed using Masson’s Trichrome stained serial sections of P1 hearts through the
AMIRA® software (Template Graphics Software, USA) as previously described. AV valve volumes were determined using the AMIRA® program.

### 3.3.6 Heart Function Measurements

Left ventricular (LV) and right ventricular ejection fraction and fractional shortening were measured using the Vevo 2100 ultrasound imaging system (Visual Sonics, Canada). Briefly, 2-dimensional images of the heart were obtained in short-axis view using a dynamically focused 40 MHz probe. The M-mode cursor was positioned perpendicular to the LV anterior and posterior walls. The LV internal end-diastolic dimension (LVIDd) and LV internal systolic dimension (LVIDs) were measured from M-mode recordings. LV ejection fraction was calculated as: \[ \text{EF} \% = \left( \frac{[(\text{LVIDd})^3 - (\text{LVIDd})^3\times100]}{\text{LVIDd}} \right) . \] Fractional shortening was calculated as: \[ \text{FS} \% = \frac{\text{LVIDd}-\text{LVIDs}}{\text{LVIDd}}\times100. \] The M-mode measurements of the left ventricular ejection fraction and fractional shortening were averaged from 3 cycles. Both left and right atrial areas were traced and calculated under the apical 4-chamber view in B-mode. AV valve E/A ratio, regurgitation and flow pattern were measured through both color flow Doppler recordings and pulsed-wave Doppler echocardiograms.

### 3.3.7 Ex vivo Heart Cultures

E12.5 WT and NOS3⁻/⁻ hearts were explanted and cultured in M199 medium (Sigma-Aldrich, USA) supplemented with 10% FBS as previous described. Hearts were stabilized for 2 hours prior to any treatment. Both WT and NOS3⁻/⁻ hearts were beating throughout the entirety of the experiment. WT and NOS3⁻/⁻ hearts were treated with 100 \( \mu \text{M} \) 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) (Sigma-Aldrich, USA) and 2 mM 8-Bromo-cGMP (Sigma-Aldrich, USA), respectively for 6 hours. Some WT and
NOS3<sup>−/−</sup> hearts were treated with recombinant TGFβ protein (10 ng/ml) for 18 hours. Hearts were then collected and mRNA levels of Snail1, TGFβ and BMP2 were analyzed by real time RT-PCR.

### 3.3.8 Real-time RT-PCR

Standard protocol was used as previous described. Briefly, total RNA was isolated from cultured fetal hearts with a RNA isolation kit (Qiagen, Canada). cDNA was synthesized using M-MLV (Invitrogen, Canada) reverse transcriptase and random primers (Invitrogen, Canada). Real-time PCR was conducted using SYBR Green PCR Master Mix as per manufacturer’s instructions (Eurogentec, USA). The oligonucleotide primers used in this study are summarized in Online Supplementary Table 1. Samples were amplified for 35 cycles using Eppendorf Real-Time PCR machine and analyzed using cycle threshold (Ct) analysis. A ratio of target gene versus 28S rRNA was used to determine the relative expression of Snail1, BMP2 and TGFβ.

### 3.3.9 Measurement of cGMP

The cGMP levels in the heart were measured using an ELISA kit according to the manufacturer’s instructions (ADI-900-014, Enzo Life Sciences, USA). Neonatal NOS3<sup>+/+</sup>, NOS3<sup>−/−</sup>, NOS3<sup>Tg</sup>/NOS3<sup>−/−</sup> and NOS3<sup>Tg</sup> hearts were used for analysis. Briefly, 10 µg of protein lysates from the isolated hearts and standards were added to the cGMP conjugated to alkaline phosphatase and an anti-cGMP antibody, and incubated for 2 hours at room temperature. This incubation allows the antibody to bind the cGMP in the sample or conjugate in a competitive manner. After 3 washes, p-nitrophenyl phosphate (pNpp) substrates were added and the plated and incubated for 1 hour at room temperature. This incubation allows the catalysis of pNpp substrate by the alkaline
phosphatase on the cGMP conjugate. The reaction was then stopped by tri-sodium phosphate solution and the optical density was read at 405 nm. The amount of signal is indirectly proportional to the amount of cGMP in the sample.

3.3.10 Endocardial Cushion Explant Culture

Endocardial cushion explants were cultured accordingly to methods previously described with minor modifications. Briefly, E10.5 NOS3+/+ and NOS3−/− hearts were planted on a 35 mm petri dish coated with 1 mg/ml collagen type I gel overnight for attachment. After attachment, the hearts were treated with 100 µl of M199 supplemented with 10% FBS and allowed 2 days for EndMT to occur. After 2 days, these hearts were imaged under phase contrast microscopy (Observer D1, Zeiss, Germany). The number of spindle-like mesenchymal cells was counted.

3.3.11 Statistical Analysis

Data are presented as mean ± SEM. Survival curves were created by the method of Kaplan and Meier and compared by log-rank test. Unpaired Student’s t test was used for 2 group comparisons. One way ANOVA followed by Student-Newman-Keuls test was performed for multi-group comparisons. P<0.05 was considered statistically significant.

3.4 Results

3.4.1 Deficiency in NOS3 impairs AV valve formation

Trichrome staining of mitral and tricuspid valves in NOS3+/+, NOS3−/−, NOS3Tg and NOS3Tg,NOS3−/− mice at P0 is shown in Figure 3.1A-J. In NOS3−/− hearts, AV valves were much smaller than those of other genotypes. The size of proximal (hinge) and distal
aspects of the valve leaflets was measured as illustrated in Figure 3.1M. Quantitative analysis of mitral and tricuspid valves show that the size (thickness) and length of the proximal and distal leaflets were significantly smaller in the NOS3+/− compared with NOS3+/+ hearts (P<0.05, Figure 3.1E-L, 3.2F, 3.3F). Cardiomyocyte-specific NOS3 overexpression (NOS3Tg,NOS3+/−) completely rescued these defects in the NOS3+/− mice (P<0.05, Figure 3.1D-F, 3.1J-L, 3.2F, 3.3F). To investigate whether the valvular defect in the NOS3−/− animals is due to premature maturation, cell density in the valves was measured.12-14 Our data showed that there was no significant difference in cell density in mitral and tricuspid valves among all groups at P0 (Figure 3.2G and 3.3G). Furthermore, the decrease in valve length was evident as early as E15.5 in both mitral and tricuspid valves (Figure 3.4A-B). These data supports a developmental defect rather than premature maturation of the AV valves in NOS3+/− mice.

The volume of the mitral and tricuspid valves was determined through 3D reconstructions (Suppl. Figure 3.2A-E and 3.3A-E). The normal mitral valve of NOS3+/+ mice had 2 distinct leaflets. However, the overall volume of the mitral valve was significantly smaller in NOS3−/− compared with NOS3+/+ mice, which was restored by cardiomyocyte-specific NOS3 overexpression in the NOS3Tg,NOS3+/− mice (P<0.01, Figure 3.2A-E). The tricuspid valve of NOS3+/+ mice had 3 distinct leaflets. Similar to the findings in the mitral valve, the overall volume of tricuspid valve was significantly decreased in NOS3−/− compared with NOS3+/+ mice, which was completely rescued by cardiomyocyte-specific NOS3 overexpression in the NOS3Tg,NOS3+/− mice (P<0.01, Figure 3.3A-E).
Figure 3.1 Malformation of mitral and tricuspid valves in NOS3\(^{-/-}\) mice, which are rescued by cardiomyocyte-specific NOS3 overexpression (NOS3\(^{Tg};NOS3^{-/-}\)). Representative tissue sections with Masson’s Trichrome staining of mitral (A-D) and tricuspid (G-J) valves in WT, NOS3\(^{-/-}\), NOS3\(^{Tg}\) and NOS3\(^{Tg};NOS3^{-/-}\) mice at P0. (E and F) Quantification of mitral valve size. (K and L) Quantification of tricuspid valve size. Anterior and posterior leaflets represent the valve leaflet closer to the septum and ventricular free wall, respectively. (M) Valve size was measured at the proximal (hinge) and distal aspects of the leaflets. White arrows represent proximal and distal measurements. Black arrow points to the hinge of the valve. Scale bar = 60 µm. Data are mean ± SEM from 5-8 mice per group. *P<0.05 vs. WT (NOS3\(^{+/-}\)), †P<0.05 vs. NOS3\(^{-/-}\) mice.
Mitral Valve

A

WT

B

NOS3\(^{-/-}\)

C

NOS3\(^{Tg}\)

D

NOS3\(^{Tg}\);NOS3\(^{-/-}\)

E

F

G
Figure 3.2 Three-dimensional (3D) reconstructions of mitral valve of WT, $NOS3^{+/−}$, $NOS3^{Tg}$ and $NOS3^{Tg};NOS3^{+/−}$ mice at P0 (A-D). 3D reconstructions were made by putting together images taken from approximately 100 serial heart sections at 5 μm using Amira software. Valve locations in the heart are shown. Whole heart images are frontal views of the heart. Isolated valve images are viewed from the left atrium into the left ventricle. E. Quantitative analysis of mitral valve volume. Data are mean ± SEM from 5 mice per group. F. Quantification of mitral valve length in P0 hearts. G. Quantification of mitral valve cell density in P0 hearts. *$P<0.01$ vs. WT. †$P<0.01$ vs. $NOS3^{+/−}$ mice. $Tg;−/−$ and $Tg;+/+$ indicate NOSTg;NOS3$^{−/−}$ and NOS3$^{Tg}$, respectively.
**Figure 3.3** 3D reconstructions of tricuspid valve of WT, NOS3\(^{+/−}\), NOS3\(^{Tg}\) and NOS3\(^{Tg}\);NOS3\(^{+/−}\) mice at P0 (A-D). 3D reconstructions were made by putting together images taken from approximately 100 serial heart sections at 5\(\mu\)m using Amira software. Valve locations in the heart are shown. Whole heart images are frontal views of the heart. Isolated valve images are viewed from the right atrium into the right ventricle. E. Quantitative analysis of tricuspid valve volume. F. Quantification of tricuspid valve length in P0 hearts. G. Quantification of tricuspid valve cell density in P0 hearts. Data are mean ± SEM from 5 mice per group. *\(P<0.01\) vs. WT. †\(P<0.01\) vs. NOS3\(^{+/−}\) mice. Tg;\(+/−\) and Tg;\(+/+\) indicate NOSTg;NOS3\(^{+/−}\) and NOS3\(^{Tg}\), respectively.
Figure 3.4 Mitral and tricuspid valve length measurements at E15.5. A. Length of anterior and posterior leaflets of the mitral valve. B. Length of anterior and posterior leaflets of the tricuspid valve. Data are mean ± SEM from 5 mice per group. *P<0.05 vs. WT. †P<0.05 vs. NOS3<sup>−/−</sup> mice. Tg;−/− and Tg;+/+ indicate NOS3<sup>Tg;NOS3<sup>−/−</sup></sup> and NOS3<sup>Tg</sup>, respectively.
3.4.2 Regurgitation of AV valves in NOS3\textsuperscript{-/-} mice

In order to investigate the functional significance of AV malformation in NOS3\textsuperscript{-/-} mice, echocardiographic imaging analysis was performed on NOS3\textsuperscript{+/+}, NOS3\textsuperscript{-/-}, NOS3Tg and NOS3Tg;NOS3\textsuperscript{-/-} mice at P0 live animals. Color flow Doppler recordings showed backflow of mitral (Figure 3.5A) and tricuspid (Figure 3.6A) valves in NOS3\textsuperscript{-/-} mice. Using pulsed-wave Doppler, the backflow velocity and duration were quantified. The velocity and duration of backflow in mitral (Figure 3.5C-D) and tricuspid valves (Figure 3.6C-D) were significantly increased in NOS3\textsuperscript{-/-} compared with NOS3\textsuperscript{+/+} mice (P<0.05). Cardiomyocyte-specific NOS3 overexpression completely rescued these abnormalities in the NOS3\textsuperscript{-/-} animals (Figure 3.5A-D, 3.6A-D).

AV valve regurgitation could potentially result in lowered cardiac function. To this end, ejection fraction and fractional shortening were determined. Our data showed that ejection fraction and fractional shortening of both left and right ventricles were significantly decreased in NOS3\textsuperscript{-/-} compared with NOS3\textsuperscript{+/+} mice (P<0.05, Figure 3.7A-D). Cardiomyocyte-specific NOS3 overexpression in NOS3\textsuperscript{-/-} mice restored ejection fraction and fractional shortening in both left and right ventricles to levels comparable to the NOS3\textsuperscript{+/+} mice. Additionally, we assessed the E/A ratio of the AV valves, which represents diastolic function or the ability of the blood to flow from the atria to the ventricles. E represents the passive filling of blood from atria to ventricles, whereas A represents the active contraction of the atria that pumps blood to the ventricles. Our results showed that no significant difference was observed between all groups in E/A ratios or the ability of the blood to flow from the atria to the ventricles (Figure 3.7E-F). Lastly, as a consequence of AV valve regurgitation, the size of atria would increase due
to the backward flow from the ventricles. As expected, the size of both left and right atrium was significantly increased in the NOS3$^{-/-}$ compared with NOS3$^{+/+}$ mice, which was restored in NOS3Tg;NOS3$^{-/-}$ mice at P0 (P<0.05, Figure 3.7G-H).
Mitral Valve

A

WT   NOS3⁻⁄⁻

NOS3ᵀᵍ   NOS3ᵀᵍ;NOS3⁻⁄⁻

B

WT   NOS3⁻⁄⁻

NOS3ᵀᵍ   NOS3ᵀᵍ;NOS3⁻⁄⁻

C

Backflow Velocity (mm/s)

D

Backflow Duration (ms)

*
Figure 3.5 Regurgitation of mitral valve in NOS3\(^{-/-}\) mice at P0. Backflow of mitral valves was determined by color (A) and pulsed-wave (B) Doppler echocardiography. Backflow during systole is indicated by arrows. WT mice had no backflow in mitral valves. However, NOS3\(^{-/-}\) mice showed significant mitral valve backflow. (C-D) Quantification of mitral valve regurgitation. Significant regurgitation was observed in NOS3\(^{-/-}\) mice, which were rescued by cardiomyocyte-specific NOS3 overexpression. Data are mean ± SEM from 6-7 mice per group. *\(P<0.01\) vs. all other groups. +/-, WT; +/-, knockout; Tg, transgenic. Tg;+/- and Tg;++/+ indicate NOS\(^{Tg}_{-/-}\);NOS3\(^{+/+}\) and NOS3\(^{Tg}_{++/+}\), respectively.
Figure 3.6 Regurgitation of tricuspid valve in NOS3<sup>+/−</sup> mice at P0. Backflow of tricuspid valves was determined by color (A) and pulsed-wave (B) Doppler echocardiography. Backflow during systole is indicated by arrows. WT mice had minor backflow in tricuspid valves. However, NOS3<sup>+/−</sup> mice showed marked tricuspid valve backflow. (C-D) Quantification of tricuspid valve regurgitation. Significant regurgitation was observed in NOS3<sup>+/−</sup> mice, which were rescued by cardiomyocyte-specific NOS3 overexpression. Data are mean ± SEM from 6-7 mice per group. *P<0.01 vs. WT (NOS3<sup>+/+</sup>), †P<0.01 vs. NOS3<sup>+/−</sup>. +/+, WT; −/−, knockout; Tg, transgenic. Tg;−/− and Tg;+/* indicate NOSTg;NOS3<sup>−/−</sup> and NOSTg, respectively.
Figure 3.7 Cardiac function determined using echocardiography at P0. (A-D) Both left and right ventricular ejection fraction and fractional shortening were significantly decreased in the NOS3⁻/⁻ mice compared with WT controls, which were rescued by cardiomyocyte-specific NOS3 overexpression. (E-F) E/A ratio showed no significant difference between all groups. (G-H) Left and right atrial size was significantly increased in NOS3⁻/⁻ mice compared with WT controls, which was rescued by cardiomyocyte-specific NOS3 overexpression. Data are mean ± SEM from 6-7 mice per group. *P<0.05 vs. WT (NOS3⁺/⁺), †P<0.05 vs. NOS3⁻/⁻. +/+ WT, +/- knockout; Tg, transgenic. Tg;/- and Tg;+/+ indicate NOSTg;NOS3⁻/⁻ and NOSTg; respectively.
3.4.3 Snail1+ and total mesenchymal cells are decreased in the endocardial cushion of NOS3−/− mice

Snail1 is a zinc finger transcriptional repressor, which inhibits adhesion molecule E-cadherin expression and induces EndMT during embryonic development.28, 29 Therefore, Snail1 is an excellent marker of mesenchymal cells in the AV cushion. To further elucidate the role of NOS3 in early AV valve formation, immunostaining of Snail1 was performed and Snail1+ mesenchymal cells were quantified at E10.5 and E12.5. Our data showed that while there were no significant changes in the overall size and volume of the AV endocardial cushion at E10.5 and E12.5 (Figure 3.8D, G), the number of Snail1 positive mesenchymal cells (brown staining in histology) and total number of mesenchymal cells within the endocardial cushion were significantly decreased in the NOS3−/− compared with NOS3+/+ hearts at E10.5 and E12.5 (P<0.05, Figure 3.8A-C, E, F). Since reduction of mesenchymal cells in NOS3−/− hearts was seen at both E10.5 and E12.5, the rest of the analysis was done in E12.5 hearts. Three-dimensional reconstruction of Snail1 expression (labeled in red) in the NOS3+/+ mice showed Snail1 completely covered the outer part of the endocardial cushion, which represents cells that are actively undergoing EndMT (Figure 3.8A). However, in the NOS3−/− mice, only limited parts of the endocardial cushion were Snail1 positive, indicating the EndMT process is impaired in the NOS3−/− mice. The number of Snail1 positive and total mesenchymal cells was completely restored by cardiac specific NOS3 overexpression (P<0.05, Figure 3.8A-C, E, F).
**Figure 3.8** EndMT is impaired in the AV cushion of NOS3\(^{+/−}\) hearts at E12.5. (A) 3D reconstructions (Left and middle columns, Red for Snail1\(^{+}\) cells; Grey for cushion) and representative Snail1 immunostaining (Right column, Brown staining indicates Snail1\(^{+}\) cells). Scale bar = 50 μm. (B) Quantification of Snail\(^{+}\) mesenchymal cell volume from 3D reconstructed AV cushions. (C) Total number of mesenchymal cells from E12.5 cushions was quantified. (D) Quantification of endocardial cushion size at E12.5 from 3D reconstructions. (E) Quantification of Snail1\(^{+}\) endocardial cushion mesenchymal cells at E10.5 from 3D reconstructions. (F) Quantification of total endocardial cushion mesenchymal cells at E10.5. (G) Quantification of endocardial cushion size at E10.5 from 3D reconstructions. N= 3-4 mice per group. *P<0.05 vs. NOS3\(^{+/+}\) or WT control. †P<0.05 vs. NOS3\(^{+/−}\) or NOS3\(^{−/−}\) control.
3.4.4 cGMP signaling is decreased in NOS3^{−/−} hearts

cGMP, a downstream signaling molecule of NOS3, was significantly decreased in the NOS3^{−/−} compared with NOS3^{+/+} hearts at P0 (P<0.05, Figure 3.9A). Cardiac specific NOS3 overexpression in NOS3^{−/−} mice increased myocardial cGMP to similar levels of NOS3^{+/+} mice (Figure 3.9A). To elucidate the precise role of NOS3 in regulating Snail1 expression, ex vivo E12.5 heart cultures were employed. The TGFβ and BMP family proteins are critical to AV valve development.15,16 Treatment with ODQ, a soluble guanylyl cyclase inhibitor significantly decreased TGFβ1, BMP2 and Snail1 mRNA expression in the WT hearts (P<0.05, Figure 3.9B-D). Conversely, NOS3^{−/−} hearts treated with 8-Bromo-cGMP, a cGMP analog showed a significant increase in TGFβ1, BMP2 and Snail1 mRNA levels (P<0.05, Figure 3.9B-D). These data suggest that NOS3 regulates the expression of TGFβ1, BMP2 and Snail1 through a cGMP-dependent pathway. To investigate if TGFβ regulates Snail1 signalling, recombinant TGFβ is used to treat WT and NOS3^{−/−} hearts. Our data showed that recombinant TGFβ protein treatment significantly increased Snail1 expression in both WT and NOS3^{−/−} hearts (P<0.05, Figure 3.9E).
Figure 3.9 cGMP regulates expression of Snail1, TGFβ and BMP2 in E12.5 hearts. (A) cGMP levels in P0 hearts. (B-D) E12.5 ex vivo heart cultures. Cultured WT and NOS3+/− hearts were treated with ODQ (100 µM), a soluble guanylate cyclase inhibitor, or 8-Br-cGMP (2 mM), a cGMP analog for 6 hrs. Snail1, TGFβ and BMP2 mRNA levels were determined by real-time RT-PCR. (E) E12.5 ex vivo heart cultures from WT and NOS3+/− mice were treated with recombinant TGFβ protein (10 ng/ml). TGFβ mRNA levels were determined by real-time RT-PCR. N=6 hearts per group for C-G. *P<0.05 vs. NOS3+/+ or WT control. †P<0.05 vs. NOS3+/− or NOS3+/− control.
3.4.5 EndMT is impaired in \textit{NOS3}\textsuperscript{-/-} endocardial cushions

To further confirm the role of NOS3 in endocardial EndMT, endocardial cushions from E10.5 embryos were dissected and cultured on collagen type I gel. Collagen type I gel was chosen to simulate the \textit{in vivo} conditions of the cardiac jelly, which the mesenchymal cells migrate into. After 2 days of culture, cells that underwent EndMT exhibited spindle-like morphology and migrated from the endocardial cushion into the collagen gel. Results showed the total number of mesenchymal cells was significantly decreased in \textit{NOS3}\textsuperscript{-/-} compared to \textit{NOS3}\textsuperscript{+/+} explants (\(P<0.05\), Figure 3.10A-B), suggesting NOS3 promotes endocardial EndMT.
**Figure 3.10** Atrioventricular (AV) endocardial cushion explant culture from E10.5

*NOS3*<sup>−/−</sup> and WT mice. (A) Spindle shaped mesenchymal cells (arrows) were formed after 24 hours of culture. Lower panel is an enlargement of the boxed area. (B) The total number of mesenchymal cells per mm of explant cushion tissue was significantly decreased in *NOS3*<sup>−/−</sup> compared to WT explants. Data are mean ± SEM from 6 explants per group. *P<0.05 vs. WT. Black scale bar = 100 µm, white scale bar = 10 µm. (C) Proposed signaling pathway by which NOS3 promotes endocardial EndMT and AV valve development. Dash lines represent signalling pathways shown by others.
3.5 DISCUSSION

The present study was carried out to examine the role of NOS3 in AV valve formation. We demonstrated for the first time that deficiency in NOS3 leads congenital AV valve defects. Additionally, lack of NOS3 also impairs endocardial EndMT. Furthermore, we showed that cardiomyocyte-specific NOS3 overexpression mice completely rescued these defects in NOS3<sup>-/-</sup> mice. In order to assess the functional significance of these defects, echocardiography was carried out on P0 live animals. Our data showed severe regurgitation of both mitral and tricuspid valves in the NOS3<sup>-/-</sup> mice. This phenotype was accompanied by reduced ejection fraction and fractional shortening and increased atrial size. Moreover, cardiomyocyte-specific NOS3 overexpression completely rescued these phenotypes. Our study suggests that NOS3 promotes endocardial EndMT and embryonic development of AV valves (Figure 3.10C).

The main function of AV valves is to prevent blood from flowing backwards from the ventricles to the atria during systole. Clinically, AV valve regurgitation can present in various forms depending on etiology and severity.<sup>29</sup> In the case of acute AV valve regurgitation, heart failure and cardiogenic shock may occur leading to death as a result of backflow from the ventricles and volume overload.<sup>30, 31</sup> Severe AV valve regurgitation, decreased heart dysfunction and increased in atrial size were observed in the NOS3<sup>-/-</sup> mice. These phenotypes highly resemble congenital AV valve insufficiency, a clinical condition with infants born with shortened or a complete lack of AV valves.<sup>32, 33</sup> However, it is possible that that mitral valve prolapse may also play a role in AV valve regurgitation seen in the NOS3<sup>-/-</sup> mice. NOS3<sup>-/-</sup> mice have coronary artery malformation which could lead to cardiac ischemia and papillary muscle dysfunction.<sup>20</sup> This could
cause AV valve regurgitation. Although we cannot eliminate the possibility of mitral valve prolapse, we saw AV valve regurgitation accompanied by a phenotypic change of the AV valve structure. Therefore, our data support the notion that AV valve regurgitation is likely a result of AV valve insufficiency. Mitral valve insufficiency is often associated with other cardiac defects such as atrial septal defects (ASD), ventricular septal defects (VSD), aortic valve insufficiency and tricuspid valve insufficiency. Tricuspid valve insufficiency, also known as tricuspid valve regurgitation, is also associated with congenital cardiac defects such as VSD and mitral valve regurgitation. These accompanying cardiac defects are very similar to phenotypes seen in the NOS3−/− mice providing an excellent animal model to study AV insufficiency. Clinically, AV valve insufficiency is a serious congenital heart disease with very limited options other than invasive open-chest surgery. The molecular mechanisms underlying this disease are still largely unknown. The present study on the role of NOS3 in AV valve formation provides a possible mechanistic explanation to AV valve insufficiency.

Snail1, a zinc finger transcriptional repressor, is critical to induction of EndMT during embryonic development. Snail1 knockout mice have defects in mesoderm formation and die around E7.5. In addition, deletion of Snai1 specifically in the epiblast results in death by E9.5 due to multiple vascular defects and increased apoptosis. To investigate the role of NOS3 in early AV valve formation, in vivo Snail1 expression and ex vivo endocardial EndMT were assessed. Our results showed the number of Snail1 positive mesenchymal cells and total number of endocardial cushion mesenchymal cells were significantly decreased in NOS3−/− compared with NOS3+/+ hearts at E10.5 and E12.5 (Figure 3.5). In addition, the total number of mesenchymal cells from E10.5
endocardial cushion cultures was significantly decreased in NOS3⁺⁻ explants (Figure 3.7A, B). These data show a reduction of endocardial EndMT in the NOS3⁺⁻ mice leading to malformation of mitral and tricuspid valves.

TGFβ is expressed at high levels in AV mesenchyme during cushion formation and the critical to EndMT and AV valve development. Blockade of TGFβ2 by neutralizing antibody inhibits EndMT in mouse heart explants and TGFβ2 knockout mice show valvular defects. BMP2 is also important for AV cushion EndMT. Nkx2.5 mediated deletion of BMP2 reduced AV cushion formation and cellularity. A classic pathway by which NO mediates its biological function is through cGMP-dependent protein kinase G (PKG) signaling. In this regard, NO has been shown to upregulate TGFβ1 and BMP2 via PKG dependent pathway. In order to study the molecular mechanism by which NOS3 promotes endocardial EndMT and AV valve development, ex vivo E12.5 heart cultures were carried out to investigate the role of NO/cGMP signaling in the expression of TGFβ1, BMP2 and Snail1. We showed that inhibition of guanylate cyclase decreased TGFβ1, BMP2 and Snail1 expression in the WT hearts while treatment with 8-Bromo-cGMP increased TGFβ, BMP2 and Snail1 mRNA levels in the NOS3⁺⁻ hearts. These findings indicate that NOS3 acts as an upstream regulator of TGFβ1, BMP2 and Snail1 through a cGMP-dependent pathway. Notably, TGFβ1 and BMP are able to induce Snail1 expression. To this end, we showed that recombinant TGFβ treatment significantly increased Snail1 expression in both WT and NOS3⁺⁻ hearts. Taken together, our data suggest that cGMP production from NOS3 upregulates TGFβ and BMP2, and promotes Snail1 expression and EndMT, leading to normal AV valve development (Figure 3.10C).
In summary, endocardial EndMT, an important process required for valvulogenesis is impaired in NOS3−/− mice at E10.5 and E12.5, leading to malformation, insufficiency and regurgitation of AV valves at P0. We anticipate that these new insights into the mechanisms of AV valve development may lead to therapeutic strategies in the prevention and possibly treatment of AV valve insufficiency.
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CHAPTER 4

Nitric oxide synthase 3 protects the aortic valve from thrombosis and calcification

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“Nitric oxide synthase 3 protects the aortic valve from thrombosis and calcification”
4.1 CHAPTER SUMMARY

Aims: Spontaneous aortic valve thrombosis is an extremely rare event but its underlying mechanisms are still largely unknown. NO produced from NOS3 maintains endothelial cell integrity and inhibits platelet aggregation. We aimed to study the role of NOS3 in maintaining normal function and morphology of the aortic valve in adult life and aging. We hypothesized that deficiency in NOS3 leads to spontaneous aortic valve thrombosis, calcification and dysfunction in aging mice.

Methods and Results: At 12 months old, 72% (13/18) of NOS3−/− mice showed severe spontaneous aortic valve thrombosis compared with WT mice (0/12). When platelets were incubated with explanted aortic valves, platelet adhesion and aggregation to the NOS3−/− valves were significantly higher compared with the WT controls. Additionally, significant regurgitation of the aortic valve during systole was observed using echocardiography in the NOS3−/− but not in WT mice. Furthermore, NOS3 deficiency resulted in significant aortic valve stenosis, calcification and fibrosis. In ex-vivo aortic valve interstitial cell cultures, inhibition of NOS by L-NAME increased calcification in WT valves. In contrast, NO donor DETA-NO significantly inhibited calcification in NOS3−/− valves.

Conclusion: Deficiency of NOS3 results in spontaneous aortic valve thrombosis, calcification and stenosis. Our study suggests that NOS3 is pivotal in maintaining the normal structure and function of the aortic valve in adulthood and aging.
4.2 INTRODUCTION

Valvular heart disease (VHD) affects over 2.5% of the general population. This number dramatically increases to more than 13.3% of individuals over the age of 75.\textsuperscript{1,2} Over the next 25 years, world population statistics project that there will be a doubling of population in individuals between the age of 75 to 84.\textsuperscript{1} By this projection, individuals over the age of 75 affected by VHD will double in the next 25 years. VHD can present in many forms, one of which is the formation of a thrombus on the aortic valve.\textsuperscript{3,4} The spontaneous formation of a thrombus on the aortic valve is extremely rare in humans with only less than a dozen of case reports in the literature.\textsuperscript{3-12} Although most of the cases were patients with tricuspid aortic valves, one case showed spontaneous aortic valve thrombosis was a result of calcific bicuspid aortic valve.\textsuperscript{5} However, aortic valve thrombosis is extremely dangerous, as it could lead to embolization, and subsequent cerebrovascular events such as stroke, myocardial infarction and death.\textsuperscript{3, 5-7, 11} Thrombosis is formed via adhesion and aggregation of platelets at the site of tissue injury followed by fibrin clot formation. Many factors have been identified to be important inhibitors of platelet activation and aggregation, such as apyrase, prostaglandin I\textsubscript{2} and nitric oxide (NO).\textsuperscript{13, 14} In addition, thrombosis formation can also be inhibited via inhibitors of coagulation cascade such as thrombomodulin, tissue factor pathway inhibitor (TFPI) and anti-thrombin III.\textsuperscript{15, 16} To our knowledge, spontaneous aortic valve thrombosis has never been studied in an animal model.

Endothelial nitric oxide synthase (NOS3) belong to a family of enzymes known as nitric oxide synthases (NOS). NOS catalyze the reaction of L-arginine and NADPH producing NO. NO is a fast acting gaseous signaling molecule that is involved in many
physiological processes including apoptosis, angiogenesis, cell proliferation and differentiation.\textsuperscript{17-19} NOS3 is important in maintaining normal cardiovascular function including vasodilation, inhibition of inflammation and vascular smooth muscle proliferation, stimulation of angiogenesis and recruitment of endothelial progenitor cells.\textsuperscript{21} In pathophysiology, NOS3 polymorphism is associated with increased risk for cardiovascular disease, increased incidence of congenital heart disease, hypercholesterolemia, hypertension, diabetes and atherosclerosis.\textsuperscript{21}

Spontaneous thrombosis of aortic valve can be seen in patients with hypercoagulative state such as polycythemia or systemic lupus erythematosus with antcardiolipin antibodies.\textsuperscript{22, 23} In addition, excessive valvular damage such as in the case of calcific aortic valve stenosis (CAVS) may induce thrombosis.\textsuperscript{5, 11} CAVS is the most common valvular disease.\textsuperscript{1} CAVS is characterized by progressive narrowing of the aortic valve resulting in aortic stenosis, calcification and thickening of the aortic valves and aortic valve regurgitation.\textsuperscript{24} Although calcified aortic valve has been associated with normal aging and valve degeneration, recent studies suggest that CAVS can be attributed to a genetic origin. Mutations in Notch1, an important factor that is involved in many developmental processes, has been shown to cause aortic valve disease similar to CAVS.\textsuperscript{25} Notch1 functions by repressing the activities of Runx2, a central transcriptional regulator in osteoblast cell fate.\textsuperscript{25} Importantly, NOS3 has been shown to act upstream of Notch1 in aortic valve interstitial cells cultured \textit{in vitro} by altering Notch1 intracellular domain nuclear localization.\textsuperscript{26} These findings suggest NOS3 may play a crucial role in aortic valve disease. The aim of our current study was to investigate the role of NOS3 in normal maintenance of aortic valve function and its role in aortic valve thrombosis and
calcification. We hypothesized that deficiency in NOS3 leads to spontaneous aortic valve thrombosis and calcification in the adult mice.

### 4.3 METHODS

#### 4.3.1 Animals

Breeding pairs of $NOS3^{-/-}$ (stock No. 002684) and wild-type C57BL/6 (WT) mice were purchased from Jackson Laboratory (Bar Harbor, Maine). A breeding program was carried out to produce neonates. Genotyping of $NOS3^{-/-}$ and $NOS3^{+/-}$ mice was performed by a polymerase chain reaction (PCR) method using genomic DNA prepared from tail biopsies. All procedures involving mouse husbandry and manipulation were in accordance with the guidelines of the Canadian Council of Animal Care and approved by the Animal Use Subcommittee at the University of Western Ontario, Canada.

#### 4.3.2 General Tissue Processing

WT and $NOS3^{-/-}$ mice were used for analysis. Hearts were isolated and fixed in 4\% paraformaldehyde, dehydrated, and embedded in paraffin. Five micro-meter transverse serial sections were mounted onto albumin/glycerin coated glass slides.

#### 4.3.3 Immunohistochemistry and Immunofluorescence

After deparaffination and rehydration, the slides were incubated for 3 minutes with 1\% Sirius red solution for fibrosis, Mason’s Trichrome staining kit for collagen and muscle. Counterstaining was performed with 0.1\% hematoxylin (Merck, Germany). Immunofluorescence was done using established protocols were used with minor modifications. Briefly, after deparaffination and rehydration of the slides, microwave antigen retrieval was applied by heating them for 10 min at 98°C in a citric acid buffer.
(10 mM in aquadest, pH 6.0). The slides were incubated overnight with either 1:100 anti-Runx2 (Santa Cruz, USA), 1:100 anti-E-selectin (Biotech, USA). Next, the goat anti-rabbit secondary antibody conjugated with GFP (1:1000, Vector Laboratories, USA) was added for 60 min in PBS. Finally, 0.1% DAPI solution was used to stain nuclei.

4.3.4 Heart Function Measurements

Left ventricular (LV) and right ventricular ejection fraction and fractional shortening were measured using the Vevo 2100 ultrasound imaging system (Visual Sonics, Canada). Briefly, 2-dimensional images of the heart were obtained in short-axis view using a dynamically focused 40 MHz probe. The M-mode cursor was positioned perpendicular to the LV anterior and posterior walls. The LV internal end-diastolic dimension (LVIDd) and LV internal systolic dimension (LVIDs) were measured from M-mode recordings. LV ejection fraction was calculated as: EF (%) = \[(LVIDd)^3 - (LVIDs)^3\]/(LVIDd)^3 × 100. Fractional shortening was calculated as: FS (%) = (LVIDd-LVIDs)/LVIDd×100. The M-mode measurements of the left ventricular ejection fraction and fractional shortening were averaged from 3 cycles. Aortic valve regurgitation and flow pattern were measured in both color flow Doppler recordings and pulsed-wave Doppler echocardiograms.

4.3.5 Platelet Adhesion and Aggregation on Aortic Valves

Aortic valves were dissected from WT and NOS3−/− 12 months old mice and cultured in M199 medium (Sigma-Aldrich, USA). Separately, the platelets were isolated from the same animals and treated with a Calcein dye (Life Technologies, Canada) for 15 minutes under room temperature. The aortic valves were then treated with WT or NOS3−/−
platelets for 1 hour at 37ºC. After the incubation period, the aortic valves were imaged under fluorescence microscope (Observer D1, Zeiss, Germany).

### 4.3.6 Aortic Valve Interstitial Cell Cultures

WT and *NOS3*−/− aortic valves were explanted from 9-12 week old adult mice and cultured in M199 medium (Sigma-Aldrich, USA). WT and *NOS3*−/− hearts were planted on a 35 mm petri dish coated with 1 mg/ml collagen type I gel for 6 days for attachment and to allow valve mesenchymal cells, which are valve interstitial cells (VICs) to migrate. After attachment, the aortic valves were treated with 300 µl of osteo-differentiation media along with L-NAME in WT aortic valves and DEAT-NO in *NOS3*−/− aortic valves. After 18 days of culture, cells were stained with 0.5% Alizarin red for calcification and imaged under phase contrast (Observer D1, Zeiss, Germany). The number of calcified mesenchymal cells was counted.

### 4.3.7 Real Time RT-PCR

Total RNA was isolated from aortic valves of 12 months adult WT and *NOS3*−/− mice with RNA isolation kit (Qiagen, Canada). cDNA was synthesized using M-MLV (Invitrogen, Canada) reverse transcriptase and random primers (Invitrogen, Canada). Real-time PCR was conducted using SYBR Green PCR Master Mix as per manufacturer's instructions (Eurogentec, USA). Samples were amplified for 35 cycles using Eppendorf Real-Time PCR machine. The expression levels of Notch1, Runx2, Sp7 and β catenin in relation to 28S rRNA as a loading control were obtained.
4.3.8 Statistical Analysis

Data are presented as mean ± SEM. Unpaired Student’s \( t \) test was used for 2 group comparisons. One way ANOVA followed by Newman-Keuls test was performed for multi-group comparisons. \( P<0.05 \) was considered statistically significant.

4.4 Results

4.4.1 Severe spontaneous aortic valve thrombosis in \( NOS3^{+/} \) mice

Severe spontaneous aortic valve thrombosis was observed in \( NOS3^{+/} \) mice at 12 months old. Representative 3-D reconstructions of normal aortic valve of a WT mouse and a thrombus on the aortic valve of a \( NOS3^{+/} \) mouse are shown in Fig. 4.1A. The thrombus was positioned on the aortic side of the valve. Next, Masson’s trichrome staining was used to assess WT and \( NOS3^{+/} \) aortic valves. WT mice showed normal cross sectional aortic section with 3 distinct valve leaflets while 27% of \( NOS3^{+/} \) mice showed only 2 valve leaflets with an enlarged aortic valve thrombosis (Fig. 4.1B). Hematoxylin and eosin staining showed the formation of the thrombus originated from the aortic valve (Fig. 4.1C). The thrombus had an intense eosin staining due to fibrin deposition (Fig. 4.1C). Quantitative analysis show 13/18 (72%) of \( NOS3^{+/} \) mice at 12 months of age had spontaneous aortic valve thrombosis, in which 100% (5/5) bicuspid aortic valve and 62% (8/13) tricuspid aortic valve \( NOS3^{+/} \) animals showed spontaneous aortic valve thrombosis. None of the WT showed any aortic valve thrombosis (\( P<0.0001 \) Fig. 4.1D). Because of hemodynamic changes, bicuspid aortic valve is associated with aortic valve calcification and thrombosis in humans. To study the specific role of NOS3 on spontaneous thrombosis and calcification, further analyses were done using mice with tricuspid aortic valves.
In order to study the role of NOS3 in platelet adhesion and aggregation on the aortic valve, the aortic valves were explanted from WT and \(NOS3^{-/-}\) 12 months old mice and incubated with platelets. After 1 hour of incubation with the valves, the amount of platelets on these valves was imaged and quantified. Our data show that WT and \(NOS3^{-/-}\) platelets were adhered significantly more to the \(NOS3^{-/-}\) compared to the WT aortic valves (Fig. 4.2A-B). Furthermore, \(NOS3^{-/-}\) platelets were more adhesive to the \(NOS3^{-/-}\) valves compared to WT valves. In addition, platelets formed clumps on the \(NOS3^{-/-}\) aortic valves, indicating platelet aggregation (Fig. 4.2A).
Figure 4.1 Severe spontaneous aortic valve thrombosis in $NOS3^{-/-}$ mice at 12 months old. (A) 3-D reconstruction of aortic valves of WT and $NOS3^{-/-}$ mice. While WT mice have three aortic valve leaflets, $NOS3^{-/-}$ mice show a bicuspid aortic valve with a spontaneous aortic valve thrombosis (Marked in red). (B) Masson’s trichrome staining of WT and $NOS3^{-/-}$ aortic valves. $NOS3^{-/-}$ mice show only 2 valve leaflets with an enlarged aortic valve thrombosis. (C) Hematoxylin and eosin staining of a large thrombus on the aortic valve from a $NOS3^{-/-}$ mouse. This is the same animal used in (B) to show that the origin of the thrombus is from the aortic valves. (D) 72% of $NOS3^{-/-}$ mice but none of the WT mice at 12 months old had spontaneous aortic valve thrombosis. *$P<0.0001$ vs. WT
Figure 4.2 Platelet adhesion and aggregation on aortic valves. (A) WT and $NOS3^{+/}$ tricuspid aortic valves were dissected from 12 months old mice and marked with calcein dye. These valves were then treated with WT or $NOS3^{+/}$ platelets to assess platelet aggregation. (B) Quantification of platelet aggregation on WT and $NOS3^{+/}$ aortic valves using fluorescence intensity. Data are mean ± SEM from 4 mice per group. *$P<0.05$ vs. corresponding WT aortic valve (AV) groups, †$P<0.05$ vs. $NOS3^{+/}$ AV+WT platelets (Pl).
4.4.2 Deficiency in NOS3 results in aortic valve regurgitation

C57BL/6 WT and NOS3\textsuperscript{-/-} mice cardiac and aortic functions were measured at 3, 5, 9 and 12 months of age. Representative images showed normal systole blood flow in WT and NOS3\textsuperscript{-/-} mice at 12 months (Fig. 4.3A). While systole blood flow was normal, diastolic blood flow showed severe aortic valve regurgitations in the NOS3\textsuperscript{-/-} mice. This was evident on both the color and pulsed wave Doppler recordings (Fig 4.3A-B). Further quantifications showed that both regurgitation velocity and duration measured by pulsed wave Doppler recordings were significantly increased (Fig 4.3C, D). Starting at 3 months, 29\% of NOS3\textsuperscript{-/-} mice showed mild to severe aortic valve regurgitation. This trend continued at 5, 9 and 12 months with 33\%, 33\% and 37\% of NOS3\textsuperscript{-/-} mice showed aortic valve regurgitation, respectively (Fig. 4.3E).

Cardiac functions measured by left ventricular ejection fraction and fractional shortening were normal at 3, 5 and 9 months in WT and NOS\textsuperscript{-/-} mice. However, left ventricular ejection fraction and fractional shortening were significantly decreased at 12 months in NOS3\textsuperscript{-/-} mice compare with age-matched WT controls (Fig. 4.4 A-C). In addition, left ventricular anterior wall thickness during diastole and systole were measured. Our data showed that there were no significant differences in the left ventricular wall thickness at any time point of the study. To further assess the NOS3\textsuperscript{-/-} hearts, LV diameter were measure. Our data showed that there is no significant difference between WT (2.16±0.035) and NOS3\textsuperscript{-/-} (2.304±0.077) LV diameter in 12 months old mice.
Figure 4.3 Severe aortic valve regurgitation in NOS3<sup>−/−</sup> mice. Regurgitation from aortic valves was determined by color (A) and pulsed-wave (B) Doppler echocardiography. Both WT and NOS3<sup>−/−</sup> mice show normal systolic outflow. However, NOS3<sup>−/−</sup> mice show significant aortic valve regurgitation during diastole (A and B, red arrows). C-D. Quantification of aortic valve regurgitation. Significant aortic regurgitation was observed in NOS3<sup>−/−</sup> but not in WT mice. Data are mean ± SEM from 6-7 mice per group. *P<0.01 vs. WT. E. The percentage of animals that showed aortic valve regurgitation at 12 months old.
Figure 4.4 Assessment of cardiac function using echocardiography. (A) Representative M-mode imaging showing decreased heart function in the $NOS3^{+/}$ mice compared with WT controls at 12 months. (B-C) Left ventricular fractional shortening and ejection fraction at 3, 5, 9, and 12 months in $NOS3^{+/}$ and WT mice. (D-E) Left ventricular wall thickness during systole and diastole in WT and $NOS3^{+/}$ mice from 3 to 12 months. Data are mean ± SEM from 5 mice per group. *$P<0.05$ vs. WT.
4.4.3 Deficiency in NOS3 results in aortic valve fibrosis and thickening of the aortic wall

To analyze collagen content, picro Sirius red staining was performed. NOS3⁻/⁻ aortic valve showed much stronger Sirius red staining than the WT at 12 months (Fig 4.5A, upper panel). Furthermore, the aortic wall in the NOS3⁻/⁻ mice was thickened compared with age-matched WT controls (Fig. 4.5A, lower panel). Quantitative analysis showed that Sirius red staining intensity and aortic wall thickness were significantly increased in the NOS3⁻/⁻ mice compared with age-matched WT controls (Fig. 4.5B, C). The total vascular smooth muscle cell numbers in the aortic wall were increased in NOS3⁻/⁻ mice. However, cell density per mm² was not significantly different between NOS3⁻/⁻ and WT controls (Fig. 4.5D). Moreover, the structure of the cells in the WT aortic wall was organized and aligned, while in the NOS3⁻/⁻, the cells within the aortic wall was disorganized and misaligned (Fig. 4.5A). Taken together, these results showed a vulnerable and disorganized aortic wall structure in the NOS3⁻/⁻ mice that is associated hypertrophy of the aortic wall cells.
Figure 4.5 Aortic valve fibrosis and aortic wall thickening in 12 months old NOS3\(^{-/-}\) mice. (A) Representative Sirius red and hematoxylin staining showing aortic fibrosis and wall thickening, respectively. (B) Quantification of fibrosis staining intensity. (C) Quantification of aortic wall thickness. (D) Quantification of cell density in the aortic wall. Data are mean ± SEM from 6-7 mice per group. *P<0.01 vs. WT.
4.4.4 Deficiency in NOS3 results in endothelial cell activation/damage

Endothelial cell activation/injury may cause valvular damage and possibly aortic valve thrombosis.\textsuperscript{5, 6} To assess whether endothelial cell activation is seen in the NOS3\textsuperscript{+/−} mice, sections were stained for the presence of E-selectin, which is an excellent marker for endothelial cell activation.\textsuperscript{30, 31} Our data showed E-selectin expression significantly increased in the NOS3\textsuperscript{+/−} mice compared with WT mice (Fig. 4.6A). Further quantification showed significantly increased number of activated endothelial cells in the NOS3\textsuperscript{+/−} mice compared with WT mice (Fig. 4.6B). To correlate this data with mRNA expression, E-selectin mRNA expression was determined in the WT and NOS3\textsuperscript{+/−} mice. Similar to protein staining, mRNA expression of E-selectin was significantly increased in the NOS3\textsuperscript{+/−} mice compared with WT mice (Fig. 4.6C). Vascular cell adhesion molecule 1 (V-CAM) and intercellular adhesion molecule 1 (I-CAM) are well known markers for endothelial cell activation.\textsuperscript{31} Our data showed mRNA levels of both V-CAM and I-CAM were significantly increased in the NOS3\textsuperscript{+/−} mice compared with WT mice (Fig. 4.6D, E).
Figure 4.6 Endothelial cell activation of the aortic valve in $NOS3^{-/-}$ mice. (A)

Representative histology images stained with E-selectin show endothelial cell activation in the $NOS3^{-/-}$ mice. (B) Quantification of E-selectin$^+$ endothelial cells in WT and $NOS3^{-/-}$ mice. (C-E) Quantification of mRNA expression of well-known markers for endothelial cell activation. Data are mean ± SEM from 4-5 mice per group. *$P<0.05$ vs. WT.
4.4.5 Deficiency in NOS3 results in severe aortic valve calcification

To assess aortic valve calcification, aortic valves from WT and NOS3<sup>−/−</sup> mice were stained for the presence of Runx2, a marker of calcification (Fig. 4.7A). Our data showed that Runx2 expression was significantly increased in the NOS3<sup>−/−</sup> mice compared to age-matched WT controls (Fig. 4.7C). In addition, B-mode echocardiography images showed brightened aortic valves, indicative of aortic valve calcification (Fig. 4.7A, red arrows). Quantitative analysis of the brightness density of the aortic valves showed that there was a significant increase in the NOS3<sup>−/−</sup> mice compared with age-matched WT controls (Fig. 4.7B).

Notch1 has been shown to be important in maintaining normal aortic valve function by repressing the transcription of Runx2. To examine if NOS3 regulates Notch1 and Runx2 expression, mRNA was isolated from 12 months old WT and NOS3<sup>−/−</sup> aortic valves. Our data showed that the Notch1 mRNA levels were significantly decreased in the NOS3<sup>−/−</sup> mice compared with WT controls (Fig. 4.7D). Furthermore, the expression of Runx2 was significantly increased in NOS3<sup>−/−</sup> mice compared with WT controls (Fig. 4.7E). To further confirm aortic valve calcification, we measured the expression of other well-known markers of calcification including Alkaline phosphatase 1, Sp7 and β-catenin, which were all significantly increased in the NOS3<sup>−/−</sup> mice compared with WT controls (Fig. 4.7F, H-I). Sox9 is a negative regulator of Runx2. Our data showed that Sox9 mRNA levels were significantly decreased in the NOS3<sup>−/−</sup> mice compared with WT controls (Fig. 4.7G).

To further study a protective effect of NOS3 against aortic valve calcification, ex-vivo cultures of aortic valve interstitial cells (VICs) were employed. The adult aortic
valve was harvested and plated on collagen to allow VICs to migrate out. These cells
were then treated with an osteo-differentiation media in the presence of a nitric oxide
synthase inhibitor L-NAME or a NO donor DETA-NO for 18 days. The cultured VICs
were stained with Alizarin red to assess calcification. Our data showed that $NOS3^{-/-}$ VICs
exhibited significantly more calcification compared with WT cells (P<0.05, Fig. 4.8A-B).
Furthermore, when WT VICs were treated with osteo-differentiation media together with
L-NAME, calcification was significantly increased compared with controls. Finally,
when $NOS3^{-/-}$ aortic valve cells were treated with osteo-differentiation media in the
presence of DETA-NO, calcification was significantly decreased compared with controls
(P<0.05, Fig. 4.8A-B). Together, these data showed a key role of NO in inhibiting VIC
calcification.
Figure 4.7 Severe aortic calcification in NOS3−/− mice. (A) Representative B-mode and histology images stained with Runx2. (B) Calcification can be analyzed from the brightness of the aortic valves in the B-mode echocardiography image (red arrows). (C) Quantification of number of Runx2 positive cells in the aortic valve. (D-I) Quantification of Notch, Runx2, AP1, Sp7, β-Catenin and Sox9 mRNA expression in WT and NOS3−/− mice at 12 months old. Data are mean ± SEM from 4-7 mice per group. *P<0.01 vs. WT
A

WT  WT + L-NAME

NOS3⁻/⁻  NOS3⁻/⁻ + DETA-NO

B

Calculated Area (μm²)

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<th>Control</th>
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* p < 0.05
† p < 0.01
Figure 4.8 Effects of NO on calcification of cultured aortic valve interstitial cells (VICs).

(A) Representative images showing cultured VICs stained with Alizarin red for calcification. WT VICs were treated the osteo-differentiation media and a nitric oxide synthase inhibitor, L-NAME. NOS3\textsuperscript{-/-} VICs were treated with osteo-differentiation media and a nitric oxide donor, DETA-NO. (B) Quantification of Alizarin red positive cells. N=3-4 cultures per group. *P<0.05 vs. WT Control. †P<0.05 vs. NOS3\textsuperscript{-/-} Control.
4.5 DISCUSSION

In the present study, we demonstrated for the first time that deficiency in NOS3 leads to spontaneous aortic valve thrombosis, regurgitation, and calcification and fibrosis. Furthermore, we showed that the normal maintenance of aortic valves is dependent on NOS3 produced NO, which inhibits VIC calcification. Additionally, we showed that NOS3 regulates Notch1 and Runx2 signalling pathway to maintain normal aortic valve function. Our study suggests that NOS3 plays a key role in the normal maintenance of aortic valves in adulthood and aging.

Aortic valve thrombosis is extremely rare in the clinic. However, patients that have this disease are often fatal due to thrombus breakage and subsequent embolism resulting in stroke and myocardial infarction. Increases in platelet aggregation are associated with thrombus formation in patients with protein S deficiency or antiphospholipid syndrome. In the present study, the NOS3−/− aortic valves were more prone to platelet adhesion and aggregation. Furthermore, NOS3−/− platelets had a significantly higher aggregation compared to WT platelets on NOS3−/− aortic valves, suggesting that NOS3 from both platelets and aortic valves contributes to inhibition of platelet aggregation on aortic valves. Thus, lack of NOS3 from both platelets and aortic valves may lead to aortic valve thrombosis in the aging NOS3−/− mice. Additional, endothelial activation/injury can be a result of normal wear and tear during aging or underlying disease condition such as calcification of the aortic valve. Normally, NO production from NOS3 protects endothelial cells from oxidative injury. Additionally, NOS3 promotes the recruitment of circulating endothelial progenitor cells, which regenerate and repair the injured endothelium. However, in the NOS3−/− mice, these
protective mechanisms are lost, resulting in thrombus formation. To our knowledge, this is the first study that links NOS3 function to the spontaneous formation of an aortic valve thrombus.

The function of aortic valves is to prevent backflow of blood from the outflow tracts into the ventricles during diastole. The normal aortic valves are made up of 3 distinct cusps which evenly distribute the mechanical stress to the valve annulus and aorta. When the even distribution of mechanical stress is disrupted such as in the case of bicuspid aortic valves, part of the valve may have a higher stress and valvular endothelial cells may get activated, leading to platelet aggregation and eventually the formation of spontaneous aortic valve thrombosis. Previous studies have demonstrated that about 30% of NOS3<sup>-/-</sup> animals show bicuspid aortic valve phenotype. In the present study, NOS3<sup>-/-</sup> mice with bicuspid aortic valve all developed spontaneous aortic valve thrombosis (100%). However, in tricuspid aortic valve NOS3<sup>-/-</sup> animals, only 61.5% developed spontaneous aortic valve thrombosis. It appears that mice with bicuspid aortic valves are possibly more susceptible to the formation of spontaneous aortic valve thrombus. However, due to a small sample size, there was no statistical difference in the incidence of aortic valve thrombosis between these 2 groups of NOS3<sup>-/-</sup> mice. Experiments are ongoing to increase the sample size of NOS3<sup>-/-</sup> mice. In CAVS, the valve cusps become progressively fibrotic and calcified. This results in stiffened valves, reduced valve movements and progressive valve narrowing resulting in aortic stenosis. If left untreated, CAVS leads to left ventricular hypertrophy and eventually congestive heart failure and death. Aortic valve calcification plays a key role in the disease progression of CAVS. Studies have shown that Notch1/Runx2 signalling limits aortic valve...
calcification.\textsuperscript{25} Notch1 is a transcription factor that is involved in many developmental processes including liver, skeleton, heart, eye, face, kidney and vasculature.\textsuperscript{41} More importantly, Notch1 mutations are associated with abnormal development of the aortic valve including bicuspid aortic valve and aortic valve stenosis.\textsuperscript{25, 42} A reduction of Notch signalling decreases epithelial to mesenchymal transition (EMT), which is necessary for proper formation of aortic valves.\textsuperscript{43} In the adult aortic valve, Notch1 signalling activates Hairy-related transcription, which physically interacts with Runx2, a master regulator of osteoblast differentiation, and represses its transcriptional activity.\textsuperscript{44, 45} Activation of Runx2 would result in osteoblast differentiation and calcification in the aortic valves. In our study, NO inhibits osteogenic differentiation of VICs, consistent with previous findings.\textsuperscript{46, 47} We further demonstrated that Notch1 expression is significantly decreased in the \textit{NOS3}\textsuperscript{-/-} aortic valves compared with age-matched WT controls, suggesting that NOS3 regulates Notch1 expression.\textsuperscript{26} Decreased Notch1 activity would increase Runx2 expression, leading to osteoblast differentiation and aortic valve calcification.

In summary, the present study demonstrates a pivotal role of NOS3 in limiting aortic valve from thrombosis and calcification, and maintaining normal aortic valve function. These beneficial effects NOS3 are likely mediated through the regulation of Notch1 and Runx2. We believe this is an excellent animal model to study spontaneous aortic valve thrombosis and the disease progression of CAVS. We anticipate that these new insights into the role of NOS3 in maintaining normal aortic valve function may lead to therapeutic strategies in the prevention and possibly treatment of spontaneous aortic valve thrombosis and CAVS.
4.6 REFERENCES


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5 CHAPTER 5

DISCUSSION

5.1 SUMMARY OF MAJOR FINDINGS AND DISCUSSION

The overall objective of this thesis was to investigate the regulatory role of NOS3 in the heart during embryonic development and adulthood. More specifically, I studied the role of NOS3 in the embryonic development of coronary artery, the formation of atrioventricular valves and the maintenance of adult aortic valves (Figure 5.1). To achieve these goals, NOS3 genetic knockout animals were used and a novel cardiac specific NOS3 overexpressing mouse was generated. Experimental approaches included in vitro cell cultures, ex vivo heart cultures and in vivo animal studies as well as the use of pharmacological inhibitors and adenoviral constructs.

In Chapter 2, I investigated the role of NOS3 in coronary artery development (Figure 5.1). I demonstrated that neonatal NOS3\(^{-/-}\) mice show severe abnormalities in coronary arteries. Furthermore, as early as E15.5, coronary artery diameters, density and volume were significantly decreased in the NOS3\(^{-/-}\) compared with WT embryos. In order to understand the molecular mechanism governing morphogenesis of the coronary arteries, expression of transcription and growth factors critical to coronary development was analyzed in E12.5 hearts. Interestingly, the expression of Gata4, VEGFa, bFGF and EPO was down-regulated in the NOS3\(^{-/-}\) compared with WT embryonic hearts. Additionally, Wilm’s tumour-1 (Wt1), a transcription factor critical for EMT and coronary artery formation, was also down-regulated and the number of epicardial derived cells (EPDC) was decreased in the NOS3\(^{-/-}\) hearts. These data suggest that NOS3 is
critical to embryonic coronary artery development. It has been shown that NOS3 promotes proliferation in many cell types.\textsuperscript{1-3} For example, NOS3 promotes neonatal cardiomyocyte proliferation by inhibiting TIMP-3 expression.\textsuperscript{1} NOS3 also promotes migration of mesenchymal stem cells towards the heart.\textsuperscript{4} Therefore it is possible that not only cell migration, but also cell proliferation of EPDCs is affected in the $NOS3^{-/-}$ mice. Thus, a combination of decreased transcription and growth factors and decreased ability of EPDC proliferation and migration into the myocardium may impair coronary artery development in the $NOS3^{-/-}$ mice.

$NOS3^{-/-}$ mice show impaired coronary artery development partially due to decreased EMT of the epicardium. EMT has also been shown to be crucial to atrioventricular valve development in the embryonic heart. Thus, in \textbf{Chapter 3}, I investigated the role of NOS3 in atrioventricular valve formation (\textbf{Figure 5.1}). I demonstrated that for the first time that deficiency in NOS3 leads to impairment of endocardial EMT and congenital AV valve defects. Furthermore, I showed that cardiomyocyte-specific NOS3 overexpression mice completely rescued these defects in $NOS3^{-/-}$ mice. In order to assess the functional significance of these defects, echocardiography was carried out on P0 live animals. Our data showed severe regurgitation of both mitral and tricuspid valves in the $NOS3^{-/-}$ mice. This phenotype was accompanied by reduced ejection fraction and fractional shortening and increased atrial size. Moreover, cardiomyocyte-specific NOS3 overexpression completely rescued these phenotypes. To investigate the role of NOS3 in early AV valve formation, \textit{in vivo} Snail1 expression and \textit{ex vivo} endocardial EMT were assessed. Our results showed the expression of Snail1 was significantly decreased in $NOS3^{-/-}$ compared with WT hearts at
E12.5. In addition, explant culture of endocardial cushions from E10.5 mice showed that the total number of mesenchymal cells was significantly decreased in NOS3−/− explants. These data show a reduction of endocardial EMT in the NOS3−/− mice leading to malformation of mitral and tricuspid valves. Our study suggests that NOS3 promotes endocardial EMT and embryonic development of AV valves. To further understand the underlying signaling mechanism, I showed that inhibition of guanylate cyclase decreased TGFβ1, BMP2 and Snail1 expression in the WT hearts while treatment with 8-Bromo-cGMP increased TGFβ, BMP2 and Snail1 mRNA levels in the NOS3−/− hearts. Both TGFβ1 and BMP have the ability to stimulate Snail1 expression. To this end, we showed that recombinant TGFβ treatment significantly increased Snail1 expression in WT and NOS3−/− hearts compared with respective controls. Taken together, our data suggest that cGMP production from NOS3 upregulates TGFβ and BMP2, and promotes Snail1 expression and endocardial EMT, leading to normal AV valve development.

In Chapter 4, I investigated the role of NOS3 in the normal maintenance of aortic valve function (Figure 5.1). I demonstrated for the first time that deficiency in NOS3 leads to severe aortic valve thrombosis, regurgitation, aortic stenosis and aortic valve calcification and thickening. Deficiency in NOS3 resulted in severe spontaneous aortic valve thrombosis at 12 months old. Histological analysis showed the formation of a thrombus originated from the aortic valve. Quantitative data showed that 72% of NOS3−/− mice at 12 months old had spontaneous aortic valve thrombosis. None of the WT showed any aortic valve thrombosis. In order to test platelet aggregation of WT and NOS3−/− mice, an ex vivo aortic valve culture explant was carried out. Our data showed that platelet aggregation was significantly increased in NOS3−/− aortic valves. In addition, the NOS3−/− aortic valves
showed clumping of platelets similar to that occurs in thrombus formation. Additionally, severe aortic valve regurgitation was observed in the NOS3−/− mice. This phenotype started at 3 months with 28.5% of NOS3−/− mice showing mild to severe aortic valve regurgitation and continued at 5, 9 and 12 months with 33.3%, 33.3% and 36.8% of NOS3−/− mice having aortic valve regurgitation, respectively. Furthermore, NOS3−/− mice had aortic stenosis starting at 3 months old. As expected, this phenotype persisted at 5, 9 and 12 months. Moreover, the aortic wall in the NOS3−/− mice was thickened compared with age-matched WT controls. To analyze calcification, aortic valves were harvested from 12 months old WT and NOS3−/− mice and stained with Runx2, a marker of calcification. My data showed that Runx2 staining was significantly increased in the NOS3−/− mice compared with age-matched WT controls. Furthermore, when WT aortic valve cells treated the osteo-differentiation media in the presence of L-NAME, a nitric oxide synthase inhibitor, calcification was significantly increased compared with controls. When NOS3−/− aortic valve cells were treated with osteo-differentiation media in the presence of DETA-NO, a nitric oxide donor, calcification was significantly decreased compared with controls. Finally I showed that the mRNA expression of Notch1 was significantly decreased in the NOS3−/− mice and Runx2 mRNA levels were significantly increased in NOS3−/− mice compared with WT controls. These results were expected since Notch1 represses the activity of Runx2 in normal aortic valves.

Taken together, my study suggests that NOS3 plays a crucial role in the normal maintenance of aortic valves during adulthood and aging.

In summary, I have demonstrated a crucial role of NOS3 in normal heart development and in the maintenance of postnatal aortic valve function. More specifically,
I showed that NOS3 is required for coronary artery development and deficiency in NOS3 leads to hypoplastic coronary arteries. Next, I showed that NOS3 promotes endocardial EMT and the formation of AV valves during embryonic heart development. Finally, I showed deficiency in NOS3 results in thrombosis formation and calcification of aortic valves, suggesting an important role of NOS3 in maintaining normal function and morphology of aortic valves. A summary of these findings and previous work using NOS3−/− mice is shown in Figure 5.2. My studies may provide important information for developing novel therapeutic strategies for the treatment of congenital heart disease and adult valvular disease.

5.2 STUDY LIMITATIONS

5.2.1 Mouse Models of Human Disease Conditions

Genetically modified mice used as models of human disease conditions are necessary in the evaluation of potential therapeutics for both ethical and practical reasons. The use of mouse models allows for the collection of tissue samples, and the control of experimental conditions that are otherwise not possible in humans. Over 99% of all mouse genes can be traced to a human homologue thus providing a genetic
Figure 5.1 Proposed signaling pathway of NOS3 on coronary artery development, atrioventricular valve formation and adult aortic valve maintenance. NOS3 promotes the expression of Wt1, Gata4, TGFβ, BMP2 and Notch1 via cGMP. Wt1 and Gata4 promote coronary artery development via EPDC migration and growth factor signalling, respectively. TGFβ and BMP2 regulate AV valve formation via activation of Snail1 and EMT in the endocardial cushion. Finally, Notch1 expression inhibits Runx2 and prevents adult aortic valve calcification. Lack of NOS3 induces endothelial activation and thrombosis of aortic valves in aging.
Figure 5.2 Summary of the biological effects of NOS3 on the heart during embryonic development and postnatal life.
system that correlates well with the human genome. Finally, the mouse genome is relatively easy to manipulate and this provides an enormous amount of possibilities for genetic modification to study various human related diseases.

This thesis utilized a variety of \textit{in vitro} and \textit{in vivo} experimental models to simulate human heart disease. \textit{In vivo}, Chapters 2 and 3 used a neonatal mouse model to study the role of NOS3 in coronary artery development and atrioventricular valve formation. Chapter 4 used an adult mouse model to study the role of NOS3 in the normal maintenance of the aortic valves. In humans, NOS3 polymorphisms with reduced enzyme activity have been shown to be correlated with congenital heart disease and coronary artery disease. In my studies, deficiency in NOS3 expression results in congenital heart defects including malformations of coronary arteries and impaired atrioventricular valve development (Chapters 2 and 3) and adult aortic valve thrombosis, stenosis and calcification (Chapters 4). Previous studies from our lab also showed deficiency in NOS3 results in congenital heart defects such as atrial and ventricular septal defects. Whole-body knockout animals and the genetic mutation or polymorphisms in human disease may not be directly comparable. In humans polymorphisms of NOS3 have been associated with congenital heart disease and coronary artery disease. These polymorphisms represent a reduction in the enzyme activity, not a complete deletion of NOS3. Therefore we would expect the severity of the phenotypes to be much greater in the animal models. Indeed, in our NOS3\textsuperscript{\textminus/\textminus} mice, up to 80\% of offspring dies within the first 5 days of life compared with the polymorphisms seen in humans which has not been associated with postnatal mortality. Next, even though in this case the phenotypes in NOS3\textsuperscript{\textminus/\textminus} animals are similar to humans, it is not certain that these phenotypes are a
primary defect caused by the deficiency of NOS3 in the heart. For example, it is possible that whole-body knockout of NOS3 leads to changes in other organ systems and through these changes causing a secondary effect on the heart leading to congenital heart disease. To address this issue, we have generated a cardiac-specific NOS3 transgenic mouse under the control of β-myosin heavy chain promoter. Expression of human NOS3 was detected only during embryonic development and specifically in the heart of NOS3Tg mice. The NOS3Tg mice were then crossed with NOS3−/− to create the NOS3Tg;NOS3−/− mouse, an animal that lacks NOS3 in all organs except the heart during embryogenesis. cGMP, a critical downstream signaling molecule of NOS3, was significantly decreased in the NOS3−/− compared with WT hearts at postnatal day 0 (P0). This animal model allowed use to study the effect of NOS3 specifically in the heart and our data support an important role of myocardial NOS3 in embryonic heart development.

For in vitro cell cultures, we isolated ventricular myocytes and epicardium derived cells (Chapter 2) and cushion cells (Chapter 3) from the embryonic hearts. We also isolated aortic valve tissues from the hearts of adult mice (Chapter 4). The use of in vitro cultures presents a problem as these cells may, under certain circumstances, respond differently than in vivo conditions. For example, the fact that in vivo ventricular myocytes are under the constant nourishment from the blood supply as well as its physical and biochemical interaction with surrounding environment simply cannot be duplicated in any in vitro cultures. In my studies, these cultures were used to overcome technical barriers in vivo. When studying the developing heart or a very specific tissue in the adult heart (aortic valves), in vivo manipulations of the tissue of interest is simply not technically possible. Therefore, we have to employ methods of in vitro culturing to
delineate the role of NOS3 in these tissues. Notably, our *in vitro* delineation of mechanisms has provided new insights on the pathogenesis of phenotypes in the NOS3−/− mice.

In summary, this thesis utilized transgenic and knockout mouse models to study the role of NOS3 in embryonic heart development and in maintaining normal aortic function during postnatal life. However, murine research also has inherent limitations in that the mouse heart is different from the human heart. For example the mouse heart beats approximately 7 times faster and 250 times smaller than the adult human heart. The most important differences in cardiac structure of human and mouse are confined to the atria. In the mouse, unlike the human counterpart, there is no extensive formation of a secondary atrial septum. Therefore, the relevance of any NOS3-mediated effects described in this thesis ultimately needs to be confirmed in humans.

### 5.2.2 Use of Pharmacological Inhibitors to Delineate Signaling Pathways

In Chapter 2 and 3 of this thesis, I used nitric oxide donors DETA-NONOate, as well as nitric oxide inhibitors such as L-NAME. These agents are easy to use, and have previously been shown to be effective in increasing or decreasing the availability of NO to cells. DETA-NONOate belongs to the family of diazeniumdiolates that release low levels of NO spontaneously at physiological conditions. The half-life of DETA-NONOate under physiological pH is approximately 20 hours and therefore, it is a slow acting prolonged NO donor that mimics NOS3 when given at low μM concentrations. L-NAME is an analogue for L-arginine. It competes with L-arginine for the substrate binding site on NOS. However, unlike L-arginine, L-NAME cannot be converted to L-citrulline and therefore NO production is inhibited. Although L-NAME is considered a
non-selective NOS inhibitor, it appears to have a higher selectivity to NOS3 than iNOS.\textsuperscript{20, 21} Therefore, the choices of the nitric oxide donor and inhibitor are based on the understanding of their functions, and studies using these agents to ensure proper dosing and selectivity. However, as is the case with all pharmacological compounds, I cannot rule out the non-specific effects of the pharmacological agents. For example, L-NAME (2 \textmu M) can also inhibit the production of prostaglandins and interleukin-1 beta.\textsuperscript{20, 21} In my studies, I used pharmacological agents as a secondary verification of genetic manipulations such as genetically altered mice. Thus I am confident that the results I obtained are indeed due to alterations of NO levels rather than non-specific effects of these agents.

5.3 SUGGESTIONS FOR FUTURE RESEARCH

The novelty of this thesis is the demonstration that NOS3 is crucial in the developing heart and maintenance of adult aortic valve function. However, there are many valuable aspects of the role of NO S3 during development still warrant further investigation.

In Chapter 2, I observed that NOS3 deficiency leads to coronary artery malformations. Coronary artery development involves many complex highly regulated steps. The epicardium first undergoes epithelial-to-mesenchymal transition (EMT) and gives rise to epicardium-derived cells (EPDCs), which migrates into the myocardium to form smooth muscle cells and fibroblasts that are essential for the development of coronary arteries.\textsuperscript{22-24} Recent clonal analysis confirmed earlier studies in avian embryos that the endothelium of the coronary vascular system derives from endothelial sprouts of sinus venosus with a minor contribution from the endocardium.\textsuperscript{25-27} Thus, endothelial
cells from sinus venosus and endocardium, and vascular smooth muscle cells and fibroblasts derived from EPDCs contribute to the formation of coronary arteries. Coronary artery development is further divided into major coronary artery development, coronary artery capillary vessel development and coronary vessels connection to the aorta. In this thesis and previous studies from our lab showed deficiency in \textit{NOS3} leads to major coronary artery malformations and decreased coronary capillary vessels development. However, we did not look at the role of NOS3 in the connection of coronary vessels to the aorta. In humans and mice, many coronary artery anomalies can occur.\textsuperscript{28-30} For example, both left main and right main coronary artery are connected to the same sinus of Valsalva of the aorta.\textsuperscript{29, 30} Whether a disruption of NOS3 mediates the abnormal connection of coronary arteries to the aorta is an interesting area to pursue for future studies.

In addition to studying the role of NOS3 in coronary artery development, one potential project could look at other vascular development in the body. There are major similarities in vessel development in the developing embryo. For example, it has been shown lungs of \textit{NOS3}-deficient mice also exhibit a paucity of distal arteriolar branches and capillary hypoperfusion, which resemble alveolar capillary dysplasia in humans.\textsuperscript{31} The finding is consistent with the role of NOS3 in capillary development and angiogenesis.\textsuperscript{32, 33}

Additionally, during development of the cardiac conduction system, Purkinje fibers receive paracrine cues from developing coronary vessels.\textsuperscript{34, 35} Inhibition of coronary artery development decreases Purkinje fiber differentiation suggesting that coronary arteries are necessary and sufficient for the induction of Purkinje fibers.\textsuperscript{34} Thus,
it is possible that coronary artery defects in $\textit{NOS3}^{-/-}$ mice may impede the development of the cardiac conduction system. \textsuperscript{34, 35} Whether lack of \textit{NOS3} impairs development of cardiac conduction system remains to be investigated.

In \textbf{Chapter 3}, I demonstrated that deficiency in \textit{NOS3} leads to congenital atrioventricular valve insufficiency and severe atrioventricular valve regurgitation. They only represent 2 of the 4 valves involved in the regulation of blood flow in the developing heart. The other 2 valves are aortic valve, which controls the blood flow between the left ventricle and outflow aorta and pulmonary valve, which controls the blood flow between the right ventricle and outflow pulmonary artery. Previous study showed the deficiency in \textit{NOS3} results in bicuspid aortic valve formation, which is a congenital malformation with only 2 instead of 3 valve leaflets. However, that study did not assess the development of the pulmonary valves. Bicuspid pulmonary valves are extremely rare and are often associated with other congenital cardiac defects. Isolated bicuspid pulmonary valve is exceedingly rare condition. Because of the extremely rare occurrence of bicuspid pulmonary valve, this condition is often under studied. Therefore, it is worthwhile to look at the role \textit{NOS3} in the development of pulmonary valves and to assess whether deficiency of \textit{NOS3} results in the formation of bicuspid pulmonary valves.

In \textbf{Chapter 4}, I showed that deficiency of \textit{NOS3} leads to calcified aortic valve stenosis. I demonstrated phenotypes of aortic valve regurgitation, stenosis, thickening and calcification of the valves. In the clinic, later stages of untreated calcified aortic valve stenosis are associated with left ventricular hypertrophy and eventually heart failure. Unfortunately we did not see these phenotypes in the 12 months \textit{NOS3}^{-/-} animals. It would be interesting to study these animals at 18 or 24 months to see if they develop left
ventricular hypertrophy and heart failure. In addition, it would be interesting to see the condition of other valves in these aged mice. More specifically, the function of the pulmonary, mitral and tricuspid valves can be assessed to see if these phenotypes are limited to the aortic valves or it may apply to all valves within the aging heart.

5.4 CLINICAL APPLICATION

Over the past decade, mutations and polymorphisms of the NOS3 gene have been identified in association with coronary artery disease.\textsuperscript{13-15} In addition, a recent study showed the common G894T polymorphism, which has reduced NOS3 function, is also a risk factor for congenital heart disease.\textsuperscript{11} This finding is consistent with the role of NOS3 in heart development in mice.\textsuperscript{12,36,37} Taken together, these findings help to establish a critical role of NOS3 in early heart development in both humans and mice. The present thesis showed for the first time a critical role of NOS3 in the developing heart and in the maintenance of adult aortic valve functions. Along with our current understanding of NOS3 mutations and polymorphisms as a risk factor, reduced NOS3 function not only puts individuals at higher risk of developing coronary artery disease and congenital heart disease,\textsuperscript{4,11,13-15} but may also affect embryonic coronary artery development, atrioventricular valve development and aortic valve dysfunction and calcification in the next generation through heritable mutations and polymorphisms in the NOS3 gene. Furthermore, we propose individuals with heritable mutations and polymorphisms in the NOS3 gene may be at risk for HCAD, atrioventricular valve insufficiency and calcified aortic valve stenosis. We anticipate that these new insights into the mechanisms of coronary artery development, atrioventricular valve development and maintenance of
adult aortic valve function may lead to therapeutic strategies in the prevention and
treatment of congenital heart disease and adult valvular disease.

5.5 CONCLUSIONS

This thesis provides the first evidence that NOS3 is crucial in the development of
the coronary artery, the formation of atrioventricular valves and the adult maintenance of
aortic valve function. While further investigation is required to determine the therapeutic
potential of NOS3 in the treatment of HCAD, atrioventricular valve insufficiency and
calcified aortic valve stenosis, it is clear that NOS3 is required in the development heart
as well as normal valvular function in the adult. Furthermore, this work has provided
valuable insight into the mechanisms by which NOS3 is involved in coronary artery
development (Chapter 2), formation of the atrioventricular valves (Chapter 3) and
maintenance of adult aortic valve function (Chapter 4). Overall my doctoral research has
broadened our understanding of NOS3 in the developing heart and regulation of normal
adult valvular function, and may contribute to the development of therapeutics for the
treatment of HACD, atrioventricular valve insufficiency and calcified aortic valve
stenosis.
REFERENCES


APPENDIX

Appendix A: UWO Animal use sub-committee protocol approvals

Qingping Feng - eSirius Notification - Annual Protocol Renewal APPROVED by the AUS 2007-011-03::5

From: [Redacted]
To: [Redacted]
Date: 03/01/2013 2:36 PM
Subject: eSirius Notification - Annual Protocol Renewal APPROVED by the AUS 2007-011-03::5
CC: [Redacted]

Western

2007-011-03::5:

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

Approval Date: 11/24/2011

The YEARLY RENEWAL to Animal Use Protocol (AUP) 2007-011-03 has been approved.

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

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

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

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

**Education and Training**

Bachelor of Medical Sciences Honors 2004/09 - 2008/05
Honors Specialization in Physiology & Pharmacology Western University, Canada

PhD in Pharmacology 2008/09 - present
Western University, Canada
Supervisor: Dr. Qingping Feng

**Employment Experiences**

Supervisor at a fast food restaurant 2004/06 - 2005/09
St Thomas, Ontario, Canada

Store Manager at Gino's Pizza 2006/05 - 2006/09
Tillsonburg, Ontario, Canada

Teaching Assistant 2008/09 - 2009/05
Holding 1hr long lecture each week for 180 students in a 3rd year pharmacology course (3550b)
London, Ontario, Canada

Teaching Assistant 2009/09 - 2010/05
Holding 1hr long lecture each week for 110 students in a 3rd year pharmacology course (3550b)
London, Ontario, Canada

Teaching Assistant 2010/09 – 2011/05
Holding 1hr long lecture each week for 120 students in a 3rd year pharmacology course (3550b)
London, Ontario, Canada

Assistant Coach 2011/02 – 2012/05
Coaching senior badminton in A.B. Lucas Secondary School
London, Ontario, Canada

Teaching Assistant 2011/09 – 2012/05
3rd year pharmacology course (3620)
London, Ontario, Canada

Teaching Assistant 2012/09 – 2013/05
3rd year pharmacology course (3620)
London, Ontario, Canada
Awards and Accomplishments

Western entrance scholarship (Over 90% average) 2004/09 - 2004/09
St Thomas, Ontario, Canada

Queen Elizabeth II Aiming For The Top Scholarship 2004/09 - 2008/05
St Thomas, Ontario, Canada

Dean's honor list for each year of study 2004/09 - 2008/05
St Thomas, Ontario, Canada

Intramural Competitive Men's Single Badminton Champion 2009/01 - 2009/05
London, Ontario, Canada

Intramural Competitive Co-ed's Double Badminton Champion 2009/01 - 2009/05
London, Ontario, Canada

Graduate Student Oral Presentation Award 2009/08 - 2009/08
Best oral presentation in graduate student category
Canadian Nitric Oxide Society, Waterloo, Ontario, Canada

Graduate Student Poster Presentation Award 2009/11 - 2009/11
Best poster presentation in graduate student category
J. Allyn Taylor International Prize in Medicine Symposium
London, Ontario, Canada

Graduate Student Poster Presentation Award 2009/11 - 2009/11
Best poster presentation in cardiovascular research category
Annual Department of Physiology and Pharmacology Research Day
London, Ontario, Canada

Graduate Student Poster Presentation Award 2010/05 - 2010/05
Best poster presentation
XXth World Congress of the International Society for Heart Research (ISHR)
Kyoto, Japan

Graduate Student Oral Presentation Award 2011/03 - 2011/03
Annual Lawson Health Research Day
Waterloo, Ontario, Canada

3-Minute Thesis Oral Presentation Award 2012/05 – 2012/05
Each participate have 3 minutes to talk about their thesis
London, Ontario, Canada
Invited Presentations

Yin Liu, Xiangru Lu and Qingping Feng. Deficiency in Endothelial Nitric Oxide Synthase Impairs Fetal Coronary Artery Development in Mice. Lawson health research day - Invited Oral Presentation Finalist, Lawson Health Research Institute, London, Ontario, Canada, March 2009.


Yin Liu, Xiangru Lu and Qingping Feng. Deficiency in Endothelial Nitric Oxide Synthase Impairs Fetal Coronary Artery Development in Mice. 5th Annual Canadian Nitric Oxide Society Conference, Waterloo, Ontario, Canada, August 2009.


Yin Liu, Xiangru Lu and Qingping Feng. Deficiency in Endothelial Nitric Oxide Synthase Impairs Fetal Coronary Artery Development in Mice. Lawson health research day, Lawson Health Research Institute, London, Ontario, Canada, March 2010.

Yin Liu, Xiangru Lu and Qingping Feng. Deficiency in Endothelial Nitric Oxide Synthase Impairs Fetal Coronary Artery Development in Mice. XXth World Congress of the International Society for Heart Research(ISHR), Kyoto, Japan, May 2010.


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Yin Liu, Xiangru Lu and Qingping Feng. Deficiency in Endothelial Nitric Oxide Synthase Impairs Fetal Coronary Artery Development in Mice. Great Mammalian Meeting, Toronto, Ontario, Canada April 2011.


Yin Liu and Qingping Feng. NOing the heart: role of nitric oxide synthase-3 in heart development. Department of Physiology and Pharmacology Seminar, October, 2012, London, Ontario, Canada


Publications


