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Pregestational Diabetes and Congenital Heart Defects: Role of Reactive Oxygen Species

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Graduate Program in Physiology

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

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Pregestational Diabetes and Congenital Heart Defects:
Role of Reactive Oxygen Species

(Thesis format: Integrated Article)

by

Hoda Moazzen

Graduate Program in Physiology, Collaborative Program of Developmental Biology

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

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The School of Graduate and Postdoctoral Studies

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London, Ontario, Canada

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ABSTRACT

Women with pregestational diabetes are at a high risk of having babies with congenital heart defects (CHDs). The mechanisms of CHD induction in the offspring of women with pregestational diabetes remain elusive. With an ever-increasing rate of diabetes in young adults, there is a pressing need to understand the underlying mechanisms and initiate effective preventative strategies. The aim of this thesis is to study the role of reactive oxygen species (ROS) signalling in heart morphogenesis, unravel molecular mechanisms of CHDs induced by pregestational diabetes and provide new insights on potential therapeutic strategies. To this end, a mouse model of pregestational diabetes induced by streptozotocin was employed. A spectrum of septal, conotruncal and coronary artery malformation were identified in the offspring of mice with pregestational diabetes. Reactive oxygen species (ROS) levels were elevated and glutathione levels were diminished in the fetal hearts of diabetic female mice. Oral treatment with an antioxidant, N-Acetylcysteine (NAC), significantly diminished the incidence of CHDs and prevented coronary artery malformation in the offspring of pregestational diabetic mice. Furthermore, pregestational diabetes reduced cell proliferation, altered transcript levels, and disrupted epithelial to mesenchymal transition (EMT) in the fetal heart of pregestational diabetic mice, which were all prevented by NAC treatment. To further study the role of basal ROS production in embryonic heart development, a NADPH oxidase Nox2 knockout mouse was utilized. We demonstrated that loss of Nox2 expression decreased ROS production, and impaired TGF-β/BMP signalling and endocardial EMT in embryonic hearts. This ultimately resulted in cardiac septum and valve defects. Thus, under normal physiological conditions ROS production
promotes heart development whereas excess ROS levels during pregestational diabetes induce CHDs. These studies show maintaining a balance of ROS levels is essential for normal embryonic heart development in mice. Furthermore, NAC may have a therapeutic potential in preventing the development of CHDs during pregestational diabetes.

**Key words:**
Reactive oxygen species, congenital heart defects, pregestational diabetes, oxidative stress, coronary artery development, endocardial cushion formation, epithelial to mesenchymal transition.
CO-AUTHORSHIP STATEMENT

The studies outlined in Chapters 2-4 were performed by Hoda Moazzen 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. Additionally, Dr. Sharon Lu assisted with troubleshooting in all experiments. Mrs. Murong Liu performed animal breeding and genotyping.

Chapter 2: This study was performed in collaboration with the following researchers:

Dr. Sharon Lu performed sample collection and blood glucose analysis for diabetic and control offspring, identified heart malformations in the offspring of pregestational diabetic mice, and assisted with technical aspects of this project; Noel Ma assisted Dr. Lu in these experiments; Tom Velenosi and Dr. Brad Urquhart measured glutathione levels by UPLC analysis (Figure 2.4); Lambertus Wisse generated the three-dimensional reconstruction of transposition of great arteries for control and pregestational diabetes mice; and Dr. Adriana Gittenberger-de Groot assisted in identifying morphological defects in postnatal mice and gave valuable comments on this manuscript.

Chapter 4: This study was performed in collaboration with the following researchers:

Dr. Yan Wu, who initially identified septal defects and performed postnatal survival analysis (Figure 4.1); and Simran Aulakh who assisted in histological analysis of embryonic and postnatal heart samples.
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LIST OF ABBREVIATIONS, SYMBOLS AND NOMENCLATURE

ALDH1a2: Aldehyde dehydrogenase 1a2
ASD: Atrial septal defects
AV: Atrioventricular
AVCD: Atrioventricular canal defect
AVSD: Atrioventricular septal defect
bFGF: Basic fibroblast growth factor
BMP2: Bone morphogenic protein 2
BMP4: Bone morphogenic protein 4
CHD: Congenital heart defect
DAB: Diaminobenzidine tetrahydrochloride
DHE: Dihydroethidium
DORV: Double outlet right ventricle
EC: Endocardial cushion
ED: Embryonic day
EMT: Epithelial to mesenchymal transition
EndMT: Endocardial epithelial to mesenchymal transition
EPDCs: Epicardial derived cells
GCL: Glutamate-cysteine ligase
GFP: Green fluorescent protein
GSH: Reduced glutathione
GSSG: Oxidized glutathione
HCAD: Hypoplastic coronary artery disease
HIF1: Hypoxia inducible factor 1
IP: Intraperitoneal
LV: Left ventricle
mTmG: membrane-Tomato/membrane-Green
NAC: N-Acetylcysteine
NOX2: NADPH oxidase 2
OFT: Out flow track
P0: Postnatal day 0
PEO: Proepicardium organ
pHH3: Phospho-histone H3
RA: Retinoic acid
RV: Right ventricle
ROS: Reactive oxygen species
STZ: Streptozotocin
TBX5: T Box 5
TGA: Transposition of Great Arteries
TGF-β: Transforming growth factor β
TOF: Tetralogy of Fallot
VEGF-A: Vascular endothelial growth factor A
VSD: Ventricular septal defects
WT: Wild type
Wt1: Wilms tumor 1
\(\alpha\text{-SMA:} \alpha\text{-smooth muscle actin}\)

*, † and †† are used for denoting statistical significance.

\(\mu\) was used for micro, \(\alpha\) was used for alpha, and \(\beta\) was used for beta.
1. CHAPTER 1

1.1 PREGESTATIONAL DIABETES

Diabetes is a major health concern worldwide. To date, 382 million people have diabetes and with an increasing rate of 10 million people annually, the number of diabetic patients is estimated to reach 592 million by 2035 [1]. According to the Canadian Diabetes Association, one in every 10 Canadian adult has diabetes. About 3 to 9 women in every 1000 pregnancies in European or North American populations have diabetes before pregnancy, which is pregestational diabetes [2-6]. Women with pregestational diabetes are at risk of having infants with congenital abnormalities in the neural tube, musculoskeletal, renal and cardiovascular systems [7,8]. Among all types of congenital abnormalities, congenital heart defects (CHDs), with the prevalence of 3-5 fold higher than non-diabetic population, are the most common type of abnormalities [9-12].

Clinical studies have shown that maternal high blood glucose levels strongly correlate with the incidence and severity of congenital defects in the offspring [13-15], whereas, good glycemic control through insulin injections reduces the incidence of congenital defects in their offspring [16-18]. Therefore, health policies have been implemented to spread awareness and encourage diabetic women to maintain good glycemic control throughout gestation by insulin treatment. Despite these measures, the rate of congenital defects in pregestational diabetes is still significantly higher than the normal population [11,19,20]. The reasons, although not completely understood, are
suggested to be due to failure in maintaining optimal glycemic levels, especially during early gestational age when organogenesis is occurring [21].

This thesis is dedicated to improve our understanding of the factors that interrupt fetal heart development due to pregestational diabetes. Specifically, I focused on the role of increased reactive oxygen species (ROS) levels in induction of CHDs and the efficiency of an antioxidant in preventing those defects that develop during pregestational diabetes. Additionally, I demonstrated that basal ROS signalling is required for embryonic heart development under normal physiological conditions. Overall, my studies show that maintaining a balance of ROS levels is essential for normal heart development in mice.

1.2 HEART DEVELOPMENT

The heart is one of the first organs to form and function in early embryonic life [22]. During development, cardiac progenitors undergo extensive proliferation, remodeling, migration and differentiation. The process of heart development is precisely regulated by a combination of transcription factors, signalling molecules and epigenetic factors [23]. The release of the genome sequence and advances in genetic tools during the past decades assisted in performing numerous studies on a variety of animal models to comprehend the sophisticated process of heart formation. Understanding the molecular mechanism of heart development is essential in order to enhance our perspective of congenital defects that arise during embryogenesis, and to identify possible therapeutic approaches to prevent those defects.
1.2.1 Origin of Heart Fields

Cardiac progenitors arise from the mesoderm germ layer [24-26]. During gastrulation, cardiac progenitor cells, which are located in the anterior primitive streak, migrate inward as part of the anterior lateral plate mesoderm (Figure 1.1 A-B) [27,28]. This migration results in formation of two lateral heart fields along the midline that express early cardiac markers. The lateral heart fields then expand across the midline to form a crescent-shaped cardiogenic plate (Figure 1.1 C). Subsequently, the two edges of the crescent-shaped structure will migrate towards the midline and join to form a tubular heart by E8 in mice (Figure 1.1 D). At this stage, slow peristaltic contractions are initiated [29,30]. The heart tube is composed of two concentric layers: the endocardium and myocardium, which are separated by a layer of extracellular matrix, termed the cardiac jelly. Structurally, the anterior end of the heart tube is linked to the arterial trunk and the posterior end is linked to the venous channels. In this system, blood drains from the venous channels into the atrium chamber then flows to the ventricular chamber and is directed out via the arterial trunk (Figure 1.1 D).

Cardiac precursors rise in a temporal and spatial manner to contribute to development of a sophisticated cardiovascular system. Extensive analysis has revealed the origin of cardiac precursors, their molecular interactions and contribution to formation of cardiovascular structures. The earliest anatomical studies marked the cardiac crescent as the origin of cardiac precursors. However, further studies revealed that disruption of the cardiac crescent did not ablate heart tube formation [31]. This, in addition to specific expression domains identified by gene trap experiments [32], led to further studies to identify other potential heart fields and subsequently, a distinct
population of cardiac progenitors that are located on the dorsal and anterior side of the cardiac crescent was identified. Thus, the precursors located at the cardiac crescent were called the primary or first heart field (FHF) and the cells located on anterior-dorsal site of cardiac crescent were called the second heart field (SHF) (Figure 1.1 C). Lineage tracing experiments demonstrated participation of FHF cells to formation of the primary heart tube. Concurrent with formation of the heart tube, SHF cells will migrate into the venous and arterial channels of the heart tube and contribute to the heart tube’s elongation [31-33]. In particular, FHF progenitors contribute to formation of the left ventricle and atria while the SHF lineage contributes to formation of the right ventricle [34], interventricular septum, the outflow tract (OFT) at the arterial pole of the heart, and portions of both atria [33] (Figure 1.1 E-F). Followed by elongation of the heart tube and cardiac looping, a group of cells arising from the cardiac neural crest will migrate from the dorsal neural tube structures through the pharyngeal arch toward the OFT of the looped heart (Figure 1.1 E-F) [23,35]. Cardiac neural crest cells will then assist in remodeling of semilunar valves, formation of cardiac vessels and septation of the OFT (Figure 1.1 G).

1.2.2 Heart looping

As the heart tube elongates the common ventricular chamber bends ventrally and dorsally and then moves to the right, thus forming a looped heart tube (Figure 1.1 E). The process of heart looping occurs during embryonic day (E) 8.25 to E10.5 in mice and day 22 to 32 in human embryos [36]. Rightward heart looping that is essential for proper orientation of the atria and ventricles is directed by left right differences in embryo. During cardiac looping the atrial end will locate dorsal-cranially and shift to the left side
Figure 1.1. Development of the mammalian heart.

Early cardiac precursors (orange) at the primitive streak (A), lateral plate mesoderm (B) and cardiac crescent (red and yellow) (C). The SHF (yellow) is located anterior and dorsal to the heart tube (D). Arrows indicate direction of SHF cells migration to the heart tube. Followed by SHF cell migration, the heart loops to the right and cardiac neural crest cells (blue) will migrate towards the OFT (E). Primary left and right atria and ventricular chambers appear (F). Further remodelling and growth of the heart will form a four-chambered heart (G). A: Anterior, P: Posterior, R: Right, L: Left. Adapted with modifications from Srivastava, et al. Cell 2006, 126(6):1037-1048.
of the body, leading to formation of the right and left atrium [37] (Figure 1.1 H). Heart looping positions the ventricles posteriorly and connected to the common OFT [38]. Although the molecular regulators of cardiac looping are not well known, differential gene expression in cells of inner and outer curvature of the looped heart suggests that cardiac looping is driven by intrinsic signalling [39]. In addition, concurrent incorporation of SHF cells into the heart tube and rightward looping indicates a possible role of SHF in cardiac looping. Followed by heart looping, development of the septal walls along the atria, ventricle and OFT is initiated to partition the heart into a four-chambered structure with distinct inflow and outflow vessels.

1.2.3 Septation of Ventricles and Atria

Septation of the atria and ventricular chambers occurs during E9.5 to E14.5 in mice and during the 5th to 7th week of gestation in humans [36,40]. The ventricular septum of the adult heart has two parts: the muscular and membranous septum. At E10.5 in mice, septation of the common ventricles initiates followed by ingrowth of muscular septum from the ventricular wall towards the base of the heart. The muscular septum does not fuse to the base of the heart at this stage, thus leaving an opening between the two ventricular chambers at the AV canal. This opening will close after formation of the membranous septum along the AV canal.

An essential process in septation of AV canal is the development of the endocardial cushion. The endocardial cushion develops followed by heart looping, when the myocardium initiates deposition of extracellular matrix in regions of AV canal and OFT. Accumulation of specialized ECM, the cardiac jelly, between the myocardial and endocardial cell layer results in formation of bulging acellular structures. Subsequently,
endocardial cells of the cardiac cushion undergo epithelial to mesenchymal transition (EndMT) and migrate toward the cardiac jelly, forming the cellular structures termed the endocardial cushion [41]. The endocardial cushion that forms between the dorsal and ventral sides of the AV canal is called the AV cushion, which will participate in atrial and ventricular septation (membranous septum), as well as AV valve formation [41]. Therefore, proper development of the endocardial cushion is a critical process for formation of a functional cardiovascular system.

Septation of the atrium chamber occurs after development of two parallel septal structures called septum primum and septum secundum [36,41]. Formation of septum primum initiates at E9.5 in mice by outgrowth of a thin interatrial membranous septum on the dorsal side towards the AV cushion (Figure 1.2 B). On the edge of the septum primum a distinct cell layer forms a mesenchymal cap[36]. The septum primum and mesenchymal cap will grow towards the AV cushion but will not fully fuse with the AV cushion. Thus, it leaves an opening between the atrial chambers at the site close to the AV cushion (Figure 1.2 C). This opening is called the foramen primum and it allows blood shunting between the atria chambers (Figure 1.2 C) [36]. The foramen primum will fully close in the fetal heart after fusion of the septum primum to the endocardial cushion at E12.5 (Figure 1.2 D). However, before closure of the foramen primum, a new foramen appears by programmed cell death (apoptosis) in the anterior side of the septum primum (Figure 1.2 C) [36]. This opening is called the foramen secundum and it enables a continuous passage of blood between the atria when the foramen primum is closed (Figure 1.2 D). At E13.5, the septum secundum grows on the right side of septum primum (Figure 1.2 E). Septum secundum is a thick muscular structure, which originates
from the anterior region of the right atrium [36]. Growth of the septum secundum covers the foramen secundum but does not fully cover the posterior inferior surface of the septum primum, leaving an opening that is called foramen ovale (Figure 1.2 F) [36]. The physiological significance of foramen ovale will be discussed in the fetal heart physiology sections.

**Figure 1.2. Formation of atrial septum.**

1.2.4 Septation of the Outflow Tract

Early in heart development, the right and left ventricles share a common OFT called truncus arteriosus. Septation of the truncus arteriosus begins at E9.5, dividing it into the aorta and pulmonary artery by E14.5 [42]. During OFT septation, a spiral septum forms in the midline of the truncus arteriosus and will grow down towards the interventricular septum and fuse with it [36,42]. This process will form an entwined structure of aorta and pulmonary artery which links the aorta to the left ventricle and pulmonary to the right ventricle. The OFT septum creates two same size arteries with dorsal-ventral orientation [42]. Loss of the dorsal-ventral patterning results in abnormal connection of aorta and pulmonary arteries to ventricular chambers. These malformations will be discussed in details in future sections.

1.2.5 Physiology of Fetal Heart

Due to the fetus’ dependence on the mother’s circulation, fetal blood circulation differs from postpartum circulation [43]. In the adult heart, deoxygenated blood flows from the inferior and superior vena cava to the right atrium and is pumped into the right ventricle. Venous blood in the right ventricle is then pumped to the pulmonary circulation via the pulmonary artery. Oxygenated blood from the lungs flows back via pulmonary veins to the left atria, pumped into the left ventricle and then ejected to systemic arteries via the aorta to distribute to all body organs [43]. In the fetus, oxygen is transferred via the placenta since the lungs are not functionally active. Thus, the fetal heart develops structural adaptations, called shunts to pass blood in different directions. An important blood shunt in the fetal heart is the ductus arteriosus that links the pulmonary artery to the
aorta. Fetal oxygenated blood received from placenta flows via the umbilical veins through the interior vena cava to the right atrium and then some is pumped to the right ventricle. When the right ventricle pumps blood towards the pulmonary artery, most of the blood is shunted through the ductus arteriosus and into the dorsal aorta due to the high-pressure resistance of the pulmonary artery. During fetal development, high oxygen tension and prostaglandin levels secreted from the placenta keep the ductus arteriosus opened [44]. The other important fetal shunt is the foramen ovale that shunts blood between the right and left atria. Increased pressure of right atria passes some of the oxygenated blood via the foramen ovale to the left atria. Following the first breath, the neonate’s lungs inflate and resistance in the pulmonary veins will significantly decrease, allowing more blood flow to the pulmonary circulation. In addition, due to reduced oxygen and increased pressure in the left atria, the septum secundum covers the foramen ovale and blocks atria blood shunt. Within hours to days after birth, with reduction of prostaglandin levels in the neonate, the ductus arteriosus is closed to avoid the mixture of oxygenated and deoxygenated blood [44]. Therefore, partitioning of the heart is functionally complete.

1.2.6 Molecular Regulators of Early Heart Development

1.2.6.1 Nkx2.5

One of the early markers of cardiomyocyte specification is Nkx2.5 (NK2 transcription factor, locus5) that is expressed in the cardiac crescent (starting at E7 in mice) and in their myogenic descendants [45,46]. Although the homologue of Nkx2.5 in Drosophila, Tinman, is essential for heart tube formation [47], Nkx2.5 expression in mice
is dispensable for heart tube formation [48], but it is specifically required for left ventricular chamber development [49]. Nkx2.5 is a DNA binding transcription factor that is expressed in both FHF and SHF during embryogenesis and demonstrates robust expression in adult cardiomyocytes as well [45,50,51]. Importantly, Nkx2.5 regulates expression of cardiac genes that determine terminal differentiation of cardiomyocytes [52]. Additionally, Nkx2.5 is robustly expressed in specialized cardiac conducting cells during embryogenesis, suggesting its possible role in development of the conduction system [53]. Nkx2.5 null mice demonstrate impaired cardiac morphogenesis including reduced myocardial growth and trabeculation [52]. Therefore, loss of Nkx2.5 results in arrest of heart development in early stages of embryonic development after heart looping [48,52].

1.2.6.2 GATA 4, 5, 6

The GATA family is a group of zinc finger transcription factors, which interact with the guanine-adenine-thymine-adenine (GATA) binding site of DNA to regulate expression of downstream genes. Six isoforms of the GATA transcription factor have been identified in vertebrates, three of which are expressed in cardiac progenitors: GATA4, GATA5 and GATA6 [54]. GATA4 is expressed in embryonic and adult cardiomyocytes [55,56]. Although, GATA4 expression is not critical for cardiac lineage specification [57], loss of Gata4 in endoderm is lethal due to impairment of cardiac morphogenesis [58,59]. Specifically, in Gata4 null mice, the lateral cardiac crescents fail to fuse, thus the heart tube will not form, leading to formation of cardiac bifida [58,59]. In addition, GATA4 directs transcriptional activation of factors that regulate cardiomyocyte proliferation and differentiation [60,61]. Furthermore, epicardial
expression of GATA4 and GATA6 are essential for development of endocardial cushion and cardiac vasculature [62,63]. GATA5 expression in the heart is identified as early as E9.5 in endocardial cells and later on in the endocardial cushion up to E12.5 in mice [64].

GATA5 plays a critical role in endocardial differentiation, aortic valve development and through interaction with GATA4 and GATA6, it contributes to formation of the OFT [64-66]. In addition to their role in regulation of cardiac development, GATA4 and GATA5 regulate cardiomyocyte proliferation cooperatively [67]. There have been limited numbers of studies on the role of GATA6 in mammalian heart development. Early investigation demonstrated that GATA6 expression is essential for development of extra-embryonic tissues at early embryonic stages, and full body knockout embryos die before implantation [68]. Studies in amphibians demonstrated that GATA6 is required for cardiac maturation, and its loss in cardiac progenitors delays cardiomyocyte terminal differentiation [69]. Notably, GATA4 and GATA6 double mutants demonstrate loss of cardiac differentiation resulting in acardia phenotype in mice [70]. With recent reports of GATA6 mutations in cases with CHDs, a more thorough analysis of GATA6 function in heart development is essential [71,72].

1.2.6.3 TBX5

TBX5 (T-box 5) transcription factor expression is observed in the cardiac crescent as early as E8 in mice, and then it is observed in the posterior part of the linear heart tube, corresponding to the region that will form the future sinus venosus and atria. TBX5 expression in the myocardium of the linear heart tube will become more robust and restricted to the left ventricle as the heart tube loops to the right. The asymmetric pattern of TBX5 expression persists up to E11.5, leaving no expression in the right ventricle and
OFT. At E13.5, expression of TBX5 will be more robust in the left superior vena cava, atria walls, septum primum and secundum [73,74]. Tbx5 null mutation disrupts expression pattern of several genes including Gata4, resulting in hypoplasia of the primitive atria, inflow tract and left ventricle, leading to arrest of cardiac development by E9.5 [75]. A 50% reduction of Tbx5 mRNA levels in Tbx5\textsuperscript{+/-} mice caused defects in formation of atrial septum secundum and primum and occasionally defects in the ventricular septum.

1.2.6.4 MEF2

Monocyte enhancer factor 2 (MEF2) is a transcription factor that binds to adenine-thymine (AT) rich DNA domains to regulate expression of muscle specific genes [76]. In the mouse MEF2 family, there are four genes: Mef1A, Mef2B, Mef2C and Mef2D. Although all of the genes are expressed during early heart development, Mef2C received considerable attention as the first Mef2 factor to be expressed in cardiac precursors and its requirement for heart looping [77,78]. SHF precursor cells residing medial to the cardiac crescent express MEF2C and this expression will continue in the OFT and right ventricle [79]. Null mutations in Mef2c results in impaired heart looping, absence of right ventricle formation and reduced expression of cardiac alpha-actin [78]. Expression of Mef2C is regulated through specific interactions of transcription factors GATA4 and ISL1 with its promoter [79].

1.2.7 Transcription Factor Interactions

Regulation of heart development by transcription factor activity is a sophisticated phenomenon. Often individuals with mutations in different transcription factors
demonstrate the same cardiac abnormality. As an example, mutations in *Gata4, Nkx2.5* or *Tbx5* may manifest as septal defects in the atria or ventricles or other CHDs that are often associated with conduction defects [23,80,81]. There has been accumulating evidence that transcription factors work in combinations to activate expression of downstream genes. As an example, functional interaction of NkX2.5 with TBX5 or GATA4, and functional interaction of GATA4 with TBX5 has been demonstrated to be essential for regulation of downstream genes controlling cardiomyocytes differentiation or septal development [82-84]. Moreover, cardiac transcription factors may activate transcriptional expression of their cofactors. As an example, GATA4 activates transcriptional expression of Nkx2.5 through interaction with the GATA binding sites on the Nkx2.5 promoter region [85] and Nkx2.5 activates expression of GATA6 through interaction with its promoter [86]. Thus, when analyzing the genetics of CHD, a sophisticated model of interacting transcription factors should be considered.

### 1.2.8 Regulators of Endocardial Cushion Development

Endocardial cushion development is critical for normal heart development and its malformation is associated with severe septal and valvular defects [87]. Formation of cellular structures in the endocardial cushion is initiated by activation of endocardial cells to undergo epithelial to mesenchymal transition (EndMT). This process has three essential stages: 1) activation of endothelial cell transformation in response to paracrine and autocrine signals from the myocardium and endocardium, respectively, 2) modulation of cell-cell adhesion and acquisition of a mesenchymal phenotype, and 3)
migration and residing in the cardiac cushion. The following is a description of growth factors and signalling molecules that are essential to form endocardial cushion structures.

EndMT is initiated upon receiving signals from the myocardium [88]. The two important classes of signalling molecules in the initiation of EndMT are bone morphogenic proteins (BMPs) and transforming growth factor β (TGF-β) that are members of the TGF-β superfamily. There are three isoforms of TGF-β: TGF-β1, 2, and 3. Around the time of endocardial cushion development, TGF-β1 shows ubiquitous endocardial expression that will gradually be restricted to endocardial cells at the AV and OFT [89]. TGF-β2 expression is restricted to the OFT and AV myocardium. TGF-β3 expression is not detectable until after formation of the cardiac cushion, and it is expressed in the myocardial as well as in endothelial and mesenchymal cells [90-92]. Due to the broad activity of the TGF-β family during early embryogenesis, null mutations in Tgf-β1 [93-95], Tgf-β2 [96] and Tgf-β3 [97,98] cause a wide range of developmental defects and early lethality in mice. Therefore, most studies on TGF-β regulation of EMT have been performed on endocardial cushion explants. These studies demonstrated that addition of either TGF-β2 or 3 or a combination of both stimulates EndMT induction in chick AV explant culture [92,99,100]. However, the significance of TGF-β isoforms varies in different species. In contrast to observations in chicks, TGF-β3 knockout mice do not exhibit overt endocardial cushion defects [97], and the role of TGF-β2 in the regulation of EndMT seems to be more prominent [101]. Differences in the temporal and spatial patterning of TGF-β2 and 3 expression suggests that TGF-β2 and TGF-β3 are sequentially and separately involved in the process of EMT in mice. While TGF-β2
mediates the initial endothelial cell-cell separation, TGF-β3 is required for the cell morphological changes that enable migration of cells into the underlying extracellular matrix [102].

The BMP family has numerous isoforms with distinct or overlapping functions. The important BMP ligands that participate in endocardial cushion formation are BMP2 and BMP4 that are expressed in the myocardium of the AV cushion in the same temporal and spatial pattern as TGF-β expression [103,104], indicating functional interaction between TGF-β and BMPs. Knockouts of Bmp2 or Bmp4 are both embryonic lethal before E9.5 making in vivo analysis difficult to perform [105,106]. However, development of antisense technology has made it feasible to examine the significance of BMPs in the regulation of EndMT [107]. These experiments demonstrated that although BMP2 is required for the initiation of EndMT, it is not sufficient to induce EMT in endocardial cells. These studies also demonstrated that BMP2 acts synergistically with TGF-β3 in the induction and regulation of EMT [108,109].

A common mediator of BMP and TGF-β signalling in the regulation of EndMT is the SMAD protein family [110,111]. Through distinct signalling pathways, both BMP and TGF-β phosphorylate and activate different family members of SMADs, which leads to nuclear localization of SMAD4 for regulation of downstream target genes such as Snail1, an important regulator of cell migration [112,113]. Another pathway that regulates Snail1 expression is the Notch1 signalling pathway that works in parallel with TGF-β in regulation of EndMT. Notch1 is expressed in endocardial cells and promotes TGF-β2-induced EMT through transcriptional activation of Snail1 expression [114,115].
Mutations in NOTCH1 or its ligand (*DELTA* or *JAGGED*) are associated with aortic valve or OFT defects, respectively [116,117].

Apart from differentiation, proliferation of endocardial cells is an essential part of endocardial cushion formation. Vascular endothelial growth factor A (VEGF-A) is an important growth factor that is expressed in endocardial and myocardial cells during early embryogenesis. During heart development, VEGF-A expression is gradually restricted to endocardial cells adjacent to the AV cushion and OFT region [118]. Myocardial overexpression of VEGF-A at E9.5 inhibits endocardial cushion formation in mice and induces CHDs [119]. Additionally, overexpression of VEGF-A in endocardial cells in mice resulted in severe abnormalities in ventricular septation and OFT remodelling, causing embryonic lethality by E12 to 14.5 [120]. NFAT (nuclear factor in activated T cells) is a transcription factor that is expressed in endocardial cells covering AV cushion, and it is crucial for endocardial cushion development [121,122]. Loss of NFATc1 in mice results in defects of endocardial cushion development, as a result of impaired cell proliferation. Importantly, VEGF is upstream regulator of this NFAT activation and nuclear localization [88,123]. Overall, these studies suggest that VEGF-A is an important regulator of endocardial cushion development and any alterations in VEGF-A dosage impairs cardiac development [88].

### 1.2.9 Morphological Aspect of Coronary Artery Development

The coronary vasculature originates from the ascending aorta at the sinus of the aortic valves to distribute oxygenated blood to the heart [124]. Coronary arteries branch from the base of the aorta and travel along well-defined paths to reach the cardiac apex.
Arteries form a vascular network to ensure an efficient supply of oxygenated blood to the myocardial cells. Deoxygenated blood is collected into capillary veins, which drain into the right atrium, inferior to the opening of superior vena cava. Structurally, coronary arteries are composed of three cell layers: endothelium, smooth muscle cells and fibroblasts. In the following, I will describe the process of coronary network formation during embryogenesis.

The coronary network in the myocardium forms during two processes: vasculogenesis and angiogenesis [125]. Vasculogenesis refers to de novo formation of the primitive vessels network and angiogenesis refers to growth and remodeling of the primitive network into a complex network. During heart development, vasculogenesis initiates after the formation of an epicardial cell layer at E10.5, followed by development of the vascular network through the process of angiogenesis. Here, I will review the morphological aspects of vasculogenesis in the embryonic heart and then describe essential molecular factors in the regulation of coronary artery formation in the heart.

Early precursors that participate in the formation of the vascular network in the heart emerge from outside of the heart field around E9.5 in mice [126], form a structure called the proepicardium organ (PEO). The PEO are a group of cells that emerge from a region close to the sinus venosus and the liver primordium. During E9.5 to E10.5 in mice, cells of the PEO migrate toward the heart and proliferate to expand over the myocardium and form the epicardial cell layer [127-129]. While epicardial cells proliferate to cover the myocardium, some of the epicardial cells undergo epithelial to mesenchymal transition (EMT) and dissociate from the epicardial cell layer to invade the myocardium [130]. Cells of epicardial origin that undergo EMT are called epicardial derived cells
(EPDCs). EPDCs primarily reside in the subepicardial space and after migration into the myocardium, they differentiate into a variety of cell types including smooth muscle cells, perivascular and interstitial fibroblasts [131-133]. The endothelial capillary network works as a scaffold for EPDCs, where they differentiate into smooth muscle cells and fibroblasts to establish the coronary artery network at E14.5. Interaction of smooth muscle cells and endothelial cells is critical since vessels will regress easily if they are not covered with smooth muscle cells [134].

The developmental origin of coronary endothelial cells has been the subject of many experimental studies and still remains a controversial issue [135-137]. Primarily, endothelial progenitors have been reported in the subepicardial space, growing into the myocardium and eventually connecting to the aortic root [138-140]. Studies on quail-chick chimera embryos demonstrated that coronary endothelial cells emerge from the PEO, where they arise as EPDCs and form primary endothelial tubes in the myocardium [136,141-143]. However, this developmental model was later challenged in epicardial lineage tracing studies of mice where very few Wt1+ and no Tbx18+ EPDCs in the myocardium obtained an endothelial cell fate [137,144]. Recent studies have provided a better understanding of endothelial cell origin by demonstrating that endothelial cells originate from the endothelial sprouts of the sinus venosus, which is located in the subepicardial space. Endothelial cells later on migrate into the subepicardial space and then the myocardium to form the endothelial cell population of coronary arteries [145,146]. This conflict appeared to be resolved after a group of researchers demonstrated that a subpopulation of cells from the PEO contribute to the formation of the sinus venosus and also cardiac endothelial cells [147]. Additionally, fate mapping and
clonal analysis demonstrated a subset of endothelial cells originated from endocardial cells [148]. Overall, coronary endothelial cells originate from a mixed precursor population. Both EPDCs and subepicardial endothelial cells contribute to the development of coronary arteries with minor contribution from the endocardium.

1.2.10 Molecular Regulators of Coronary Artery Development

Transcription factors, signalling molecules and growth factors that regulate formation of the epicardium and EPDCs are numerous [149]. Signals that regulate coronary artery development arise from two sources: epicardial and myocardial cells. Epicardial and myocardial cells signal to each other to coordinate formation of the vascular network and growth of the myocardium [150]. It is believed that hypoxia is the initial signal that drives vasculogenesis. Hypoxia inducible factor 1 (HIF1) is a transcription factor that is expressed during hypoxia and oxygen levels will alter its stabilization or degradation [151]. HIF1 will activate transcriptional expression of several pathways including genes that are involved in angiogenesis, cell proliferation, apoptosis, glucose metabolism, and cell survival [152,153]. HIF1 transcription factor is composed of two isomers called α and β, which dimerize for transcriptional activity [154]. Functional studies in embryos revealed high activity of HIF1α in the regulation of vasculogenesis. Reduced HIF1α expression impairs normal coronary artery development [155,156]. One of the downstream targets of HIF1α that has a pivotal role in coronary vasculature formation is Wilms tumor-1 (Wt1) [157]. During cardiac development, Wt1 is a key regulator in the formation of the PEO and epicardial cells [158]. Conditional
knock out of \textit{Wt1} in epicardial cells results in loss of coronary artery development and fetal lethality between E16.5 to E18.5 [159].

A prominent downstream target of Wt1 in the regulation of epicardial EMT are the transcriptional factors Snail1 and Slug that belong to the family of zinc finger transcription factors [159-162]. Slug expression is detected in the PEO, epicardial cells as well as epithelial and mesenchymal cells in the avian heart [163]. Snail1 expression is not detected in the PEO but it is present in epicardial and subepicardial cells at E10.5-12.5 as well as in EPDCs at E14.5-16.5 in mice [164]. Snail1 and Slug promotes EMT through repressing the expression of E-cadherin, a transmembrane protein that forms cellular junctions and inhibit cell migration. However, in comparison to Snail, Slug demonstrates less ability to inhibit \textit{E-cadherin} expression [162,165].

Retinoic acid (RA), the active derivative of vitamin A, has a prominent role in the development and growth of the myocardium and coronary vasculature [166]. In particular, RA signalling mediates vasculature development by maintaining myocardial and epicardial cell adhesion that is essential for proper signalling between the myocardium and epicardium. As well, RA signalling promotes epicardial EMT, EPDCs differentiation and cardiomyocyte proliferation through activation of bFGF secretion from the myocardium [167,168]. RA depletion during early embryogenesis impairs growth and development of the early heart tube and also diminishes \textit{Gata4} transcriptional expression [169,170]. In addition, depletion of RA signalling during vasculogenesis impairs epicardial cell layer formation, myocardial growth and formation of the vascular network [168,171]. Notably, Wt1 and retinaldehyde dehydrogenase (RALDH2), the enzyme that catalyzes a critical step in RA production, have an epicardial expression
pattern [158,172]. Recent studies demonstrated that Wt1 regulates epicardial expression of RALDH2 [173,174] in the regulation of coronary vasculogenesis in mice [175]. Hence, through various mechanisms, RA regulates myocardial growth and vascular development of cardiac progenitors in the heart.

1.3 CONGENITAL HEART DEFECTS

As discussed in the previous sections, heart development is ruled by precise regulation of gene expression and signalling molecules. Modifications to gene expression during heart development will lead to abnormalities in heart structure and impair heart function. The morphological defects that occur during heart development in embryos are called congenital heart defects (CHDs). The incidence of CHDs in the general population is 1-5% [176,177]. With advances in cardiac repair surgeries, many children born with CHDs will survive to adulthood, thus, genetic factors that induce cardiac defects will be more likely to be passed onto the next generation [178]. Based on recent reports, the prevalence of CHDs in infants born to mothers with CHDs is 2-20% [179]. This raises a public health concern and demonstrates a critical need to understand the underlying molecular mechanism of CHD and development of preventative and therapeutic strategies.

1.3.1 Genetics and Environmental Basis of CHDs

Abnormalities in cardiovascular development are caused by inherited or non-inherited factors [180,181]. Inherited factors are presented as chromosomal disorders, Mendelian syndrome or non-syndromal single gene mutations. Chromosomal disorders
account for 8 to 10% of CHDs, Mendelian syndrome accounts for 3 to 5% of CHDs and non-syndromal single mutation accounts for a very small population of CHDs [181]. Thus, at present, only about 15% of CHDs can be attributed to a genetic factor while the cause of the other 85% of CHDs still remains unknown. Non-inherited factors refer to environmental conditions that increase the prevalence of CHDs in infants. These factors include maternal alcohol consumption, nicotinic smoke, and maternal illness such as pregestational diabetes [12]. Collectively, it is speculated that the majority of CHDs have a multifactorial origin, referring to a combination of genetic and environmental factors.

1.3.2 Types of Congenital Heart Defects

CHDs studied in this thesis can be divided into four groups: septal, conotruncal, valvular and coronary artery malformations. Septal defects include malformation of the atrial or ventricular septum. Conotruncal defects include tetralogy of Fallot (TOF), pulmonary atresia, double outlet right ventricle (DORV), and dextro-transposition of great arteries (d-TGA or TGA). Cardiac valve defects include malformation of aortic or atrioventricular valves, and coronary artery malformations include formation of hypoplastic coronary arteries. The following section is a description of the origin of these defects and their genetic basis.

1.3.3 Atrial Septal Defects

Atrial septal defect (ASD) occurs when there is a failure in formation of the septum between the right and left atria, thus blood shunts between atrial chambers. ASD is a common type of CHDs with a worldwide prevalence of 1.6 per 1000 live births
ASD reduces heart function but it is not a cyanotic type of heart defect, meaning that it does not cause blue coloring of the skin or lips. This is due to the fact that the higher pressure in the left atrium will direct oxygenated blood into the deoxygenated right atrium. ASDs may occur in the septum primum or septum secundum. Defects in formation of the septum primum occur as a result of failure in fusion of the septum primum to the endocardial cushion. Thus, ASD primum defects may rise due to decreased cell proliferation of the septum primum or abnormal development of endocardial cushion [183]. Secundum ASD is persistence of the foramen ovale, which may occur as a result of inadequate growth of septum secundum or excess apoptosis in septum primum. As discussed in previous sections, foramen secundum forms by apoptosis (Figure 1.1). However, excessive apoptosis in the septum primum could result in an oversized foramen secundum that does not cover up the septum secundum membrane. Holt Oram syndrome, associated with TBX5 mutation and DiGeorge syndrome, associated with TBX1 mutation, are both chromosomal abnormalities that result in ASD secundum defects [184]. In addition, non-syndromal familial single mutations in NKX2.5 [185], TBX5 and GATA4 [84] are also associated with secundum defects. Recent reports indicated a molecular interaction of GATA4, NKX2.5 and TBX5, which is required and critical for formation of the atrial septum [186].

1.3.4 Ventricular Septal Defects

As mentioned before, the ventricular septum is composed of membranous and muscular structures. The membranous septum is a small part of the ventricular septum and is located beneath the AV valves at the base of the heart. The muscular septum
extends from the apical side of the ventricles to the membranous septum. Septal defects can occur either in the muscular or membranous part of the ventricular septum [187]. The worldwide prevalence of ventricular septal defect (VSD) is 2.6 per 1000 live births [182]. Interruption in formation of the membranous septum or fusion of the membranous to muscular ventricular septum results in an opening in the septum that allows blood flow communication between oxygenated and deoxygenated blood of the two ventricles. Mutations in NNX2.5 and GATA4 genes have been reported in cases with VSD [188-193]. Additionally, Holt-Oram syndrome or TBX5 mutation is associated with VSD [84]. Notably, human genetic studies reported a GATA4 mutation that impairs its functional interaction with TBX5 and is associated with familial incidence of VSD [84,194]. This data implies that functional interaction of GATA4, NNX2.5 and TBX5 may be essential for ventricular septum development.

1.3.5 Atrioventricular Septal Defects

Atrioventricular septal defect (AVSD) or atrioventricular canal defect (AVCD) is occurrence of an ASD at the site of ostium primum that is higher than the AV valves accompanied by a membranous VSD [195]. Structurally, AVSD is caused by malformation of the endocardial cushion and is often associated with abnormal AV valve (mitral and tricuspid) formation. AVSD has a 48% postnatal mortality rate [196] and with the prevalence of 0.24-0.31 per 1000 live births, it is considered a rare type of CHD [197,198]. The incidence of AVSD in neonates with trisomy 21 (Down’s Syndrome) is 17% [199,200]. The genes involved in non-syndromic AVSD include NNX2.5, GATA4, and TBX5 [201-205]. Although the mechanism underlying this defect is not fully
understood, pre-gestational diabetes significantly increases the risk of both syndromic [206] and non-syndromic complete AVSD [207].

1.3.6 Tetralogy of Fallot

The most common cyanotic defect is tetralogy of Fallot (TOF), which originates due to malformation of the aorticopulmonary septum. This defect includes four elements: pulmonary stenosis, right ventricular hypertrophy, overriding aorta and VSD. Pulmonary stenosis occurs as a result of unequal partitioning of the OFT. Thus, the aorta is wider and the pulmonary artery is narrowed. The second element of TOF is overriding aorta, which is the opening of the aorta over both ventricles. Overriding aorta occurs due to misalignment of the OFT septation which slightly shifts the OFT to the right and it straddles over both ventricles. The third element of TOF is a VSD, which is due to abnormal formation of the interventricular septum. The forth element is hypertrophy of the right ventricle, which is a physiological response to the narrowed pulmonary artery. Since the pulmonary artery is narrowed it has a higher resistance and the right ventricle must pump with more force to overcome the resistance, resulting in right ventricle hypertrophy. The consequence of TOF is shunting of the deoxygenated blood from the right ventricle to left ventricle through the VSD and misaligned aorta.

TOF is reported in 3.3 per 10,000 live births and corresponds to 6.8% of all types of CHDs [182,208]. TOF may occur as a result of anomalies in chromosomes 8 and 22 [209,210]. In chromosome 22 anomaly (DiGeorge syndrome), the $TBX1$ gene lies within the deleted region. Additionally, $TBX1$ polymorphism has been reported in individuals with OFT defects [211]. Single gene mutations in $JAG-1$, a Notch1 ligand, $VEGF-a$,
NKX2.5, GATA4 and its cofactor FOG2, have been identified in individuals with TOF [181,212-214]. However, these mutations are identified in a limited population of TOF patients. As an example, only 1-4% of TOF patients have a mutation in the NKX2.5 gene [215,216] and only 1.3% of TOF patients have mutations in FOG2 [215,217]. Additionally, despite a report on VEGF-a mutation in patients with TOF, a recent meta-analysis of a large population of TOF patients did not find a significant contribution of VEGF-a mutation to the incidence of TOF [218]. Therefore, our knowledge on genetic regulators of TOF is still limited. The environmental risk factors of TOF include maternal alcohol consumptions, RA (vitamin A) deficiency and maternal illnesses such as diabetes, rubella and phenylketonuria [181].

1.3.7 Dextro-Transposition of the Great Arteries

In the normal heart, the aorta originates from the left ventricle and the pulmonary artery originates from the right ventricle. Septation of the aorta and pulmonary artery occurs after formation of a spiral shaped aorticopulmonary septum. In the case of dextro-transposition of great arteries (d-TGA or TGA), the aorticopulmonary septum does not form in a spiral shape and instead it forms a straight septum [182,219]. This division causes the aorta to open into the right ventricle and the pulmonary trunk to open into the left ventricle. Consequently, in the case of TGA, the pulmonary artery transfers oxygenated blood to the lungs and aorta caries deoxygenated blood to the body. Prenatally, this defect will not cause death due to the placenta blood transfer. Babies with TGA are born alive due to the opening of the ductus arteriosus, which is a vessel that links the aorta and pulmonary and allows mixture of oxygenated and deoxygenated
blood. However, corrective surgery is required otherwise death will occur. The worldwide prevalence of TGA is 3.5 per 10,000 live births [182,219]. Mutations in \( NKX2.5 \) and \( NODAL \) are associated with non-syndromal causes of TGA. Environmental conditions such as maternal diabetes is a risk factor for TGA [181].

1.3.8 Double Outlet Right Ventricle

Double outlet right ventricle (DORV) occurs as a result of disruption in remodeling of the OFT. In this type of defect, both the aorta and pulmonary are connected to the right ventricle. Hearts with DORV have an obligatory VSD for circulation of blood between the heart chambers. The incidence of DORV is estimated to be 1.6 per 1000 live births [213]. A small percentage of DORV is associated with chromosomal abnormalities such as trisomy 18 [181,220] or chromosomal deletion of 22q11 [221-223]. Single gene mutations in \( NKX2.5 \) or \( GATA4 \) have a rare contribution to the incidence of DORV [220,224]. Mutation in the GATA4 cofactor, FOG2 was identified in 15% of patients with DORV [215]. Additionally, DORV demonstrated strong association with pregestational diabetes [225].

1.3.9 Hypoplastic coronary artery disease

Hypoplastic coronary artery disease refers to abnormal development of coronary arteries, with marked reduction in the length or diameter of one or more major branches [226]. This condition may severely affect the heart’s function and results in sudden cardiac arrest in young or middle age adults [227-231]. Hypoplastic coronary artery is a silent disease and usually unexpectedly identified during angiography [232] or the autopsy following sudden cardiac death [233]. The incidence of coronary artery
anomalies in patients that are suspected for coronary artery obstruction is about 1% [234-237]. Our knowledge on the genetic roots or environmental conditions that result in hypoplastic coronary artery disease is limited. A recent study showed that deficiency in nitric oxide release from endothelial nitric oxide synthase during heart development diminishes Wt1 and Gata4 transcript levels and results in formation of hypoplastic coronary arteries and consequently myocardial infarction in neonatal mice [238].

1.4 MECHANISM OF CHDS IN PREGNANCIES AFFECTED BY DIABETES

As discussed in previous sections, maternal diabetes before pregnancy elevates the risk of CHDs in the offspring. A combination of in vivo and in vitro experiments has been performed to enhance our understanding of the factors that induce congenital anomalies in pregnancies affected by diabetes. Primarily, studies in the 80’s demonstrated that the teratogenic effect of diabetes is related to elevation of blood glucose levels and not decreased insulin levels [239-243]. In vitro, addition of high glucose interrupted embryonic development and induced major embryonic malformations [244-246]. Supplementation of insulin-treated serum from diabetic mice to embryonic cultures reduced congenital malformations but did not prevent the risk of abnormalities [247,248], suggesting that serum of diabetic mice contains other teratogenic factors that were not eliminated by insulin treatments.

The predominant form of glucose transportation in the placenta and embryonic heart tissue is glucose transporter 1 (GLUT1) [249,250] that is independent of insulin. Females with hyperglycemia will have excess glucose that is freely transported across the
placenta and metabolized in the embryonic tissue [251]. Increased glucose levels will increase glycolysis and this will lead to increased levels of NADH and FADH2, which are the electron donors for the electron transport chain of mitochondria. Increased mitochondria respiratory chain activity leads to increased oxygen consumption and ATP production. As a result of this process, superoxide generation that is a by-product of ATP production will be increased [252,253]. Interaction of superoxide with other molecules generates a combination of reactive oxygen species (ROS) that are highly reactive and can damage DNA or proteins. Additionally, diabetes is often associated with attenuation of antioxidant bioavailability. Overall, this condition that is defined as excess ROS generation and reduction of antioxidants levels is called oxidative stress and it is detrimental to embryonic growth, organogenesis and remodelling [254].

In physiological conditions, excess ROS is depleted by an antioxidant defense system including catalase, superoxide dismutase and glutathione peroxidase. Glutathione peroxidase is an important antioxidant enzyme in depletion of ROS. In this enzymatic reaction, glutathione peroxidase breaks down H$_2$O$_2$ to H$_2$O and O$_2$ with the consumption of reduced glutathione (GSH) and converts it to oxidized glutathione (GSSG) [255]. In diabetic conditions, due to increased activity of glutathione peroxidase [256], reduced GSH are mostly oxidized. In addition, de novo GSH synthesis is diminished [256] that leads to reduced antioxidant capacity. Thus, H$_2$O$_2$ will be converted to hydroxyl radicals (OH$^\cdot$), which will cause extensive damage to molecular and cellular structures including DNA and proteins [254]. Overall, several studies have suggested a strong correlation of hyperglycemia with oxidative stress and congenital defects in embryos. However, the
specific molecular mechanism that leads to these defects and the potential therapeutic strategies remained elusive.

Experiments in the late 90’s indicated that oxidative stress plays a major role in induction of birth defects. Early evidence on the role of oxidative stress in induction of birth defects appeared following reports that indicated supplementation of superoxide scavengers to embryonic culture media inhibited malformation induced by hyperglycemia [257]. Specifically, addition of ROS scavengers such as superoxide dismutase (SOD), catalase and glutathione peroxidase inhibited hyperglycemia-induced malformation. In addition, another study confirmed that overexpression of SOD led to reduced fetal malformations in the offspring of diabetic mice [258]. Furthermore, extensive studies revealed that addition of antioxidants such as vitamin E, C, butylated hydroxytoluene and GSH-ester to diabetic animals or high glucose cultures reduced the rate of malformations [256,259-263]. Therefore, these findings strongly implied that diabetic induced malformations were caused by oxidative stress and decreasing oxidative stress in embryos could be an important therapeutic approach.

### 1.4.1 ROS Regulation of Growth and Development

ROS refers to the derivatives of oxygen free radicals including \( \text{O}_2^- \) (superoxide anion), \( \text{HO}^- \) (hydroxyl radical) and \( \text{H}_2\text{O}_2 \) (hydrogen peroxide) that are produced from different cellular sources under physiological or pathophysiological conditions [264]. The main sources of ROS production are: 1) mitochondria respiratory mechanism, 2) NADPH oxidases, and 3) enzymatic activities such as xanthine oxidase and cytochrome p450. In embryos, a prominent source of intracellular ROS production is NADPH oxidase.
NADPH oxidase is a holoenzyme complex, with 7 isoforms of its catalytic subunit including NOX 1-5 and DOUX 1-2 [265]. NADPH oxidase enzyme complex is mostly localized on the plasma membrane. The produced superoxide may pass through aquaporin channels in the form of H₂O₂ and enter the cell to be involved in different biological reactions.

ROS can mediate a variety of fundamental cellular process such as proliferation, differentiation, migration and apoptosis [266]. It is suggested that ROS functions as a classical signalling molecule, where the levels of ROS release determines the cell’s response. As an example, excess ROS levels induce cellular apoptosis and it is detrimental to embryonic growth [267-269], whereas, low or basal ROS levels mediates expression of genes required for differentiation [270] or proliferation [271,272].

1.4.2 Glutathione and Its Precursor N-Acetylcysteine

Glutathione (GSH) is an important intracellular antioxidant with high intracellular concentration. Structurally, glutathione is a tripeptide that is composed of glutamate, cysteine and glycine [255]. GSH is the product of two enzymatic reactions that are mediated by two enzymes: glutamate-cysteine ligase (GCL) and GSH synthase. In the first reaction, cysteine is conjugated with glutamate by enzymatic activity of GCL and in the second reaction, glycine is added to glutamate-cysteine by enzymatic activity of GSH synthase (Figure 1.3). GCL activity is regulated by feedback inhibition of its final product, GSH [273]. Thus this metabolic reaction is considered as a rate-limiting step for GSH synthesis. Cysteine is required for GSH production. A major source of cysteine in humans is through diet or catabolism of proteins or methionine in the liver.
Figure 1.3. Glutathione synthesis

Through an enzymatic reaction, N-Acetylcysteine (NAC) is converted to amino acid cysteine (Cys). Addition of amino acids glutamate (Glu) and glycine (Gly) to cysteine will produce glutathione peptide (GSH). Adapted with modifications from Rushworth and Megson, Pharmacol Ther 2013, 141(2), 150-9.
A precursor of cysteine is N-Acetylcysteine (NAC). Structurally, NAC is composed of an acetyl group attached to the nitrogen atom of the amino acid cysteine (Figure 1.3). Due to its small molecular size, NAC can cross the placenta and cell membrane. Intracellulary, NAC exerts its antioxidant effects by reacting with OH’ and O_2’ [274,275]. In addition, most of the NAC is deacetylated to cysteine and utilized to replenish GSH levels [276,277]. NAC has a long history in clinical use to overcome glutathione deficiency in several disease conditions including: pulmonary disease, acetaminophen (paracetamol) toxicity, neurodegenerative disease and nephropathy [278-282]. Overall, these studies reported that the protective effects of NAC are through attenuation of oxidative stress and apoptosis. Moreover, experimental analysis demonstrated that the teratogenic effects of diabetic serum or high glucose in rat embryonic development are prevented by NAC supplementation [283,284]. In addition, NAC improved neural crest cell migration in a high glucose environment, in vitro and in ovo [285,286]. Thus, these studies suggest that NAC may exert beneficial effects in protecting embryos against oxidative stress induced in diabetic conditions.

1.5 OVERALL HYPOTHESIS AND AIMS

Pregestational maternal diabetes is associated with 3-5 fold increased risk of CHDs in neonates [9-12]. With the ever-increasing population of young adults with diabetes, our understanding of the molecular causes of birth defects and the therapeutic strategies to prevent these defects is critical. Accumulating evidence demonstrated excess ROS production and a reduced antioxidant defense system lead to oxidative stress in
embryos affected by pregestational diabetes [256-263]. The main goal of this thesis project is to understand the role of ROS during cardiac morphogenesis in diabetic and non-diabetic pregnancies. The overall hypothesis of this thesis is that maintaining proper ROS balance is required for expression of critical factors that regulate cardiac morphogenesis.

The first aim of this thesis was to characterize CHDs in the offspring of mice with pregestational diabetes and to further investigate the state of oxidative stress and molecular mechanisms that are impaired in fetal heart during diabetic embryopathy. We further investigated therapeutic strategies to prevent CHDs in the offspring of mice with pregestational diabetes. In Chapter 2, I hypothesized that pregestational diabetes elevates oxidative stress in embryonic heart, alters cardiac gene expression levels and induces CHDs in neonates. Furthermore, I hypothesized that treatment of diabetic mice during gestation with an antioxidant, NAC, will reduce oxidative stress in the embryonic heart, and restore gene levels to prevent the development of CHDs in the offspring. A streptozotocin (STZ) induced mouse model was employed to characterize CHDs in the offspring. We characterized a broad range of defects including septal and conotruncal defects in the offspring of diabetic females. Both glutathione and ROS levels were determined in fetal hearts. In addition, cell proliferation and apoptosis, as well as expression levels of growth factors and transcription factors were assessed. Furthermore, the effect of NAC treatment in diabetic dams during gestation on fetal heart development was studied.

CHDs may include abnormal formation of coronary artery structures, leading to null coronary artery or hypoplastic coronary artery disease [238]. Formation of coronary
arteries is pivotal for myocardial development and abnormalities in coronary artery structure elevates the risk of sudden cardiac death in youth [227]. Whether pregestational diabetes impairs coronary artery development is not known. Therefore, in Chapter 3, I examined the formation of coronary vasculature in the neonate born to mice with pregestational diabetes. I hypothesized that pregestational diabetes interrupts formation of coronary arteries by elevating ROS levels, altering expression levels of genes essential for vasculogenesis and interrupting migration of progenitor cells that participate in formation of coronary arteries. To test this hypothesis, formation of the capillary network and coronary arteries was assessed in the fetal and neonatal hearts of the offspring using histology and immunostaining. To study epicardial contribution to coronary artery formation, Wt1-mediated lineage tracing was performed. The expression of genes essential for vasculogenesis and epicardial cell migration and proliferation were determined. Additionally, effects of NAC treatment in the diabetic dams on their offspring’s coronary vasculature development, ROS production, gene expression and epicardial cell migration and proliferation were investigated.

Excess ROS levels are detrimental to embryonic growth and development [287]. However, the role of basal ROS levels in regulating heart development and growth remained elusive. In Chapter 4, I investigated the role of basal ROS production in embryonic heart development. I hypothesized that physiological levels of ROS are essential for normal heart development in mice. To address this hypothesis, I employed mice with a deletion in the *NADPH oxidase Nox2* locus, which abolishes ROS production from NOX2 source. Heart morphology was examined for congenital heart defects in neonates. *In vivo* cell proliferation, apoptosis and cell density of endocardial cushions
were assessed. Additionally, the number of cells undergoing EndMT was studied *ex vivo*. Furthermore, the role of the ROS/TGF-β/BMP signalling pathway in the regulation of EndMT was investigated. Thus, this thesis sought to examine the impact of pathological and physiological levels of ROS production in heart morphogenesis and their molecular mechanisms. The ultimate goal of my studies was to provide novel insights in developing therapeutic strategies in the prevention of CHDs in the offspring of pregestational diabetes.
1.6 REFERENCES


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212. Lambrechts D, Devriendt K, Driscoll DA, Goldmuntz E, Gewillig M, Vlietinck R, Collen D, Carmeliet P: Low expression VEGF haplotype increases the risk for


CHAPTER 2

N-Acetylcysteine prevents congenital heart defects induced by pregestational diabetes

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“N-Acetylcysteine prevents congenital heart defects induced by pregestational diabetes”

† Equal Contribution
2.1 CHAPTER SUMMARY

Pregestational diabetes is a major risk factor of congenital heart defects (CHDs). Glutathione is depleted and reactive oxygen species (ROS) production is elevated in diabetes. In the present study, we examined whether treatment with N-Acetylcysteine (NAC), which increases glutathione synthesis and inhibits ROS production, prevents CHDs induced by pregestational diabetes. Female mice were treated with streptozotocin (STZ) to induce pregestational diabetes prior to breeding with normal males to produce offspring. Some diabetic mice were treated with N-Acetylcysteine (NAC) in drinking water from E0.5 to the end of gestation or harvesting of the embryos. CHDs were identified by histology. ROS levels, cell proliferation and gene expression in the fetal heart were analyzed. Our data show that pregestational diabetes resulted in CHDs in 58% of the offspring, including ventricular septal defect (VSD), atrial septal defect (ASD), atrioventricular septal defects (AVSD), transposition of great arteries (TGA), double outlet right ventricle (DORV) and tetralogy of Fallot (TOF). Treatment with NAC in drinking water in pregestational diabetic mice completely eliminated the incidence of AVSD, TGA, TOF and significantly diminished the incidence of ASD and VSD. Furthermore, pregestational diabetes increased ROS, impaired cell proliferation, and altered Gata4, Gata5 and Vegf-a expression in the fetal heart of diabetic offspring, which were all prevented by NAC treatment. Treatment with NAC increases GSH levels, decreases ROS levels in the fetal heart and prevents the development of CHDs in the offspring of pregestational diabetes. Our study suggests that NAC may have therapeutic potential in the prevention of CHDs induced by pregestational diabetes.
2.2 INTRODUCTION

Congenital heart defects (CHDs) are the most common birth defects affecting up to 5% of live births in the general population [1]. Pregestational diabetes mellitus, either type 1 or type 2, increases the risk of CHDs in infants by 3–5 fold compared to non-diabetic pregnancies [2-6]. With an increase in the number of young adults having diabetes mellitus [7, 8], the incidence of pregestational diabetes and CHDs caused by maternal diabetes may further increase, with significant social and economic consequences.

Although factors responsible for the high incidence of CHDs in pregestational diabetes are still not fully understood, evidence suggests that oxidative stress may play a role [9, 10]. For example, the antioxidant capacity of the developing embryo is limited [11, 12], and reactive oxygen species (ROS) production is exacerbated as the expression and activities of major ROS scavenging enzymes including superoxide dismutase and glutathione peroxidase are decreased during maternal diabetes[13-15]. In addition, maternal hyperglycemia diminishes the level of an important intracellular antioxidant, glutathione (GSH) [16, 17], which places the developing embryo in an extremely vulnerable state to oxidative stress.

N-Acetylcysteine (NAC) is a thiol-containing antioxidant agent and can cross the placenta [18]. The main biological effect of NAC as a precursor of cysteine is to replenishing cellular GSH levels and to preserve the thiol redox status. Additionally, NAC also reacts with hydroxyl radical (\'OH), nitrogen dioxide (\'NO2) and thiol radicals to reduce oxidative stress [19]. Furthermore, NAC treatment in vitro and in ovo diminishes high glucose-induced developmental defects in mouse and chicken embryos.
In the present study, we hypothesized that NAC treatment in diabetic mice during gestation diminishes ROS production and prevents the development of CHDs in their offspring. To test this hypothesis, a pregestational diabetes mouse model was established to closely simulate CHDs in patients with pregestational diabetes. We demonstrated that NAC treatment in pregestational diabetic mice decreased ROS levels and improved cell proliferation during embryonic heart development, and prevented CHDs in the offspring of diabetic mice.

2.3 METHODS

2.3.1 Animals

C57BL/6 wild type mice were purchased from Jackson Laboratory (Bar Harbor, Maine). A breeding program was implemented to generate fetal and postnatal mice. Animals in this study were handled in accordance with the Guide for the Care and Use of Laboratory Animals, published by the U.S. National Institutes of Health (NIH publ. no. 85–23, revised 1996). Use of animals was approved by the Animal Use Subcommittee at the University of Western Ontario, Canada.

2.3.2 Induction of Diabetes Mellitus and N-Acetylcysteine Treatment

Eight week old C57BL/6 female mice were treated with streptozotocin (STZ, 80 mg/kg body weight, IP, Sigma, Canada) for 3 consecutive days. STZ was dissolved in sodium citrate (pH 4.0) and mice treated with sodium citrate were served as controls. Non-fasting blood glucose levels were determined one week after STZ injection using a glucose meter (OneTouch Ultra2, LifeScan, Canada, Burnaby, BC, Canada). Mice with blood glucose levels higher than 11 mmol/l were bred to normal adult males. Mating was
verified by observation of a vaginal plug, which was counted as day E0.5 of pregnancy. A subset of control and diabetic mice received 4 mg/ml N-Acetylcysteine (NAC, 1 g/kg body weight/day) in drinking water [21] from E0.5 to the end of gestation or harvesting of the embryos. Non-fasting blood glucose levels were monitored in all groups during gestation. Water and food intake of pregnant mice was measured and normalized to body weight.

2.3.3 Histological Analysis

Heart morphology was analyzed in postnatal day 0 (P0) mice and cell proliferation was analyzed by phospho-histone H3 (pHH3) staining in E12.5 hearts. Briefly, the mouse thorax was fixed in 4% paraformaldehyde overnight, dehydrated in ethanol, embedded in paraffin and serially sectioned into 5-µm sections. Heart sections were stained with hematoxylin/eosin (H/E) and images were captured using a light microscope (Observer D1, Zeiss, Germany). Images were taken on every 25 μm of the heart and the three-dimensional visualization of heart structures was reconstructed using AMIRA® program (FEI, Hillsboro, OR, USA). To analyze cell proliferation and apoptosis, heart sections were immunostained using anti-pHH3 (phospho S10) antibody (1:500, Abcam, Toronto, ON, Canada) and anti-cleaved caspase-3 antibody (1:300, Cell Signaling), respectively, followed by incubation with biotinylated goat anti-rabbit IgG (1:500, Vector Laboratories, Burlingame, CA, USA). Signals were visualized by 3-3’diaminobenzidine tetrahydrochloride (Sigma-Aldrich Chemie, St. Louis, MO, USA). Counterstaining was performed with modified Mayer’s hematoxylin (Thermo Scientific, Waltham, MA, USA). The number of pHH3+ cells from at least 3 individual heart sections per sample was quantified and normalized to areas of the myocardium.
2.3.4 Analysis of Superoxide Levels

Embryonic heart tissues were harvested at E12.5 in all four groups. Frozen samples were cut into 10-μm sections using a cryostat (CM1950, Leica, Germany). Superoxide levels were assessed by incubation of heart sections with 2 μM dihydroethidium (DHE) (Invitrogen Life Technologies, Burlington, ON, Canada) for 30 minutes in a humidified and light protected chamber in room air at 37 °C [22]. DHE fluorescence signals were detected using a fluorescence microscope (Observer D1, Zeiss, Germany). For analysis of superoxide levels, 5–8 images of each heart sample were captured using fixed exposure time for all groups. The intensity of fluorescence signals per myocardial area was quantified using AxioVision software. A limitation of this assay is that the oxygen level was not adjusted to that of the embryonic hearts in vivo [23].

2.3.5 Real-time RT-PCR Analysis

Total RNA was extracted from individual E11.5 fetal hearts using RNeasy Mini kit (Qiagen, Burlington, ON, Canada) as per manufacturer’s instructions. One hundred nanograms of total RNA were used to synthesize cDNA using M-MLV reverse transcriptase. Real-time PCR was conducted using EvaGreen qPCR MasterMix (Applied Biological Materials, Vancouver, BC, Canada). Specific primers were designed for Nkx2.5, Gata4, Gata5, Tbx5, Tgf-β1, Vegf-a, Mef2c, cyclin D1 and Bmp4 (Table 2.1). Samples were amplified for 35 cycles using Eppendorf Realplex (Eppendorf, Hamburg, Germany). Values were normalized with 28S ribosomal RNA. The mRNA levels in relation to 28S rRNA were determined using a comparative CT method [24].
<table>
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<td>R: CTGGTGCGTCACCGGATGTC</td>
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2.3.6 Glutathione Levels in Fetal Hearts

Briefly, E14.5 fetal hearts were washed in PBS and snap frozen in liquid nitrogen. Heart samples were homogenized in 6% sulfosalicylic acid and 1 mM EDTA then centrifuged at 8,000 g for 5 minutes at 4°C. Total and reduced glutathione were assessed using a modified ultra-performance liquid chromatography (UPLC) method [25, 26]. N-isoamyl alcohol was added to 50 µL of supernatant fraction of all samples. To determine total glutathione, thiols were reduced with NaBH4 followed by the addition of HCl to adjust the pH to approximately 8.0, and then derivatized by the addition of 25 mM monobromobimane. To determine reduced glutathione, the pH of the sample was raised to approximately 8.0 with NaOH and the samples were immediately derivatized as described above. Following derivatization, the pH of all samples was decreased to approximately 4.0 with glacial acetic acid and 5 µL was injected onto a Kinetex C18 column (50 X 2.1 mm, 1.7 μm particle, Phenomenex, Torrance, CA) which was maintained at 40°C in a Waters AQUITY UPLCTM H-Class System. The mobile phase consisted 5% acetonitrile and 95% 5 mM KH2PO4 with 0.1% triethylamine, pH 4.0. The derivatized glutathione was detected by a Waters ACQUITY UPLC® fluorescence detector with the excitation set to 390 nm and the emission set to 480 nm.

2.3.7 Statistical Analysis

Data are presented as means ± SEM. Statistical analysis was performed using two-way analysis of variance (ANOVA) followed by Bonferroni post test. The incidence of congenital malformations was analyzed by Chi-square test. P < 0.05 was considered statistically significant.
2.4 RESULTS

2.4.1 Effects of NAC on Maternal Blood Glucose Levels, Litter Size and Mortality at Birth

One week after STZ injection, female mice with blood glucose levels higher than 11 mM were set up to breed with normal males. Diabetic mice had significantly higher blood glucose levels at the time of vaginal plugging (E0.5) compared to controls ($P < 0.001$, Figure 2.1 A). Additionally, time to vaginal plugging that lead to successful pregnancy was 10 times longer in the diabetic compared to control mice (26.7±6.1 vs. 2.7±0.9 days, $P < 0.01$), indicating decreased fertility rate in diabetic females. From E0.5 to E18.5 of gestation, blood glucose levels of diabetic mice were progressively increased but not significantly altered by NAC treatment (Figure 2.1 A). Average litter size of neonates from diabetic mice at P0 was significantly less than controls (Figure 2.1 B). Additionally, neonates from diabetic mice had 46% mortality rate at birth ($P < 0.001$, Figure 2.1 C). Administration of NAC in diabetic dams improved litter size of the offspring ($P < 0.05$) and diminished their mortality at birth to 11.5% ($P < 0.001$). The body weight of neonates born to diabetic mice was significantly lower compared to controls at P0 ($P < 0.001$, Figure 2.1 D). NAC treatment did not affect body weight of neonates in control mice, but significantly improved the body weight of the diabetic offspring ($P < 0.001$, Figure 2.1 D). Food and water intake was significantly higher in the diabetic females, which was decreased by NAC treatment (Figure 2.2 A-B).
Figure 2.1. Blood glucose levels of pregnant mice, litter size, mortality and body weight of neonates at P0.

A) Non-fasting blood glucose levels before mating (basal), and E0.5 to E18.5 after pregnancy in STZ-treated and control female mice with and without NAC treatment (n = 7-10 mice per group). B) The offspring litter size. C) Mortality of neonates at birth. The numbers in brackets indicate the number of death to total. D) Body weight of the offspring at birth (n = 27-29 per group). *P < 0.001 vs. untreated control, †P < 0.001 vs. untreated diabetes. Data are means ± SEM.
Water and food intake was significantly higher in diabetic females. NAC treatment decreased food and water intake of diabetic females. Data are means ± S\text{EM}. N=12-15 females per group. *$P<0.001$ vs. untreated control, †$P<0.001$ vs. untreated diabetic and control NAC.

Figure 2.2. Water and food consumption in female mice.

A-B) Water and food intake was significantly higher in diabetic females. NAC treatment decreased food and water intake of diabetic females. Data are means ± SEM. N=12-15 females per group. *$P<0.001$ vs. untreated control, †$P<0.001$ vs. untreated diabetic and control NAC.
2.4.2 Effects of NAC on Incidence of CHDs in Diabetic Offspring

Pregestational diabetes resulted in 58% CHDs in the offspring at postnatal day 0 (Table 2.2). The majority of the defects were malformations of the septum with 31% atrial septal defect (ASD, Figure 2.3 B) and 40%, ventricular septal defect (VSD, Figure 2.3 C). In addition, 7% of diabetic offspring showed atrioventricular septal defect (AVSD, Figure 2.3 D). Defects in the outflow tract included 13% double outlet right ventricle (DORV, Figure 2.3 G) and 7% transposition of great arteries (TGA, Figure 2.3 I). Furthermore, 5% of diabetic offspring showed tetralogy of fallot (TOF) with pulmonary stenosis (Figure 2.3 E), overriding aorta and VSD associated with right ventricle hypertrophy (Figure 2.3 F). Treatment with NAC in diabetic mice during gestation significantly reduced the incidence of CHDs to 16% (Table 2.2). Specifically, NAC treatment decreased incident of ASD and VSD to 14% and 12%, respectively. Also, outflow tract formation and remodeling were improved as the rate of DORV was reduced to 7% while AVSD, TGA and TOF were fully rescued by NAC treatment in the diabetic mice (Table 2.2). Craniofacial defects were also observed in diabetic embryos (5%, Figure 2.4 J). However, none of the controls or NAC treated groups showed any craniofacial defects.
Table 2.2. The rate of congenital heart defects in the offspring of diabetic and control females with and without N-Acetylcysteine (NAC) treatment.

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<td>3 10</td>
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<td>19 30.6**</td>
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<td>5 11.6††</td>
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<tr>
<td>DORV</td>
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<td>3 4.8</td>
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</tbody>
</table>

Data were analyzed by Chi-square test. * $P < 0.05$, ** $P < 0.001$ vs. untreated control, †$P < 0.05$, ††$P < 0.001$ vs. untreated diabetes. ASD, atrial septal defect; VSD, ventricular septal defect; AVSD, atrioventricular septal defect; TGA, transposition of great arteries; DORV, double outlet right ventricle; TOF, Tetralogy of Fallot.
**Figure 2.3. Congenital defects in the diabetic offspring at P0**

(A) A normal heart of control offspring. The offspring of mice with pregestational diabetes show (B) atrial septal defect (ASD), (C) ventricular septal defect (VSD), (D) atrioventricular septal defect (AVSD), (E and F) tetralogy of Fallot (TOF). (E) *pulmonary stenosis (PS), (F) VSD, overriding aorta (OA) marked by an arrow, and RV hypertrophy (RVH), (G) double outlet right ventricle (DORV), and (I) transposition of great arteries (TGA). Panels (H) and (I) are 3D-reconstructed images of a normal heart and a TGA, respectively. In panel (H) the aorta (red) is connected to the right ventricle (RV) while the pulmonary artery (PA in blue) is connected to LV. This defect is associated with a VSD. (J) Craniofacial defect in diabetic offspring. RA: right atria, LA: left atria, LV: left ventricle. Arrows indicate ASD or VSD. Scale bar is 200 µm.
2.4.3 Effects of NAC on Glutathione and ROS levels in the Fetal Heart of Diabetic Mice

Fetal glutathione levels were measured in E14.5 hearts. Diabetic offspring has significant reductions in total glutathione, GSH and GSSG levels compared to non-diabetic controls ($P < 0.01$, Figure 2.3 A-C). The GSH/total glutathione ratio was lower while GSSG/total glutathione ratio was higher in fetal hearts from diabetic mice ($P < 0.05$, Figure 2.3 D and E). NAC treatment completely restored total glutathione and GSH levels in the offspring of diabetic mice ($P < 0.001$, Figure 2.3 A and B), leading to higher GSH/total glutathione ratio and lower GSSG/total glutathione ratio ($P < 0.01$, Figure 2.4 D-E). NAC treatment in non-diabetic females during gestation had no effect on total glutathione levels but decreased GSSG levels, and consequently GSH/total glutathione ratio was increased ($P < 0.05$, Figure 2.4 D). To examine the effects of NAC on ROS levels, dihydroethidium (DHE) was employed as a probe to assess superoxide generation in fetal hearts at E12.5. Elevated DHE fluorescence reading in the fetal heart of diabetic offspring indicated excess superoxide levels, which was significantly inhibited by NAC treatment ($P < 0.05$, Figure 2.4).
Figure 2.4. Measurement of intracellular glutathione levels in fetal hearts at E14.5

A) Total glutathione levels, B) Reduced glutathione (GSH) levels, C) Oxidized glutathione (GSSG) levels, D) GSH to total glutathione ratio, and E) GSSG to total glutathione ratio. Data are means ± SEM, n = 7–9 samples per group. *P < 0.05, **P < 0.001 vs. untreated control, †P < 0.001 vs. untreated diabetes.
Figure 2.5. Analysis of superoxide in fetal hearts at E12.5 using dihydroethidium (DHE) as a probe

A) Representative images of DHE staining in the LV myocardium of fetal hearts. B) Quantification of DHE fluorescence intensity. Data are means ± SEM, n = 5–6 samples per group. *P < 0.05 vs. untreated control, †P < 0.05 vs. untreated diabetes. Scale bar is 20 µm.
2.4.4 Effects of NAC on Cell Proliferation and Apoptosis in the Embryonic Heart from Diabetic mice

Proper cell proliferation and apoptosis are essential for normal embryonic heart development [27, 28]. Using phosphorylated histone H3 (pHH3) as a marker, we analyzed cell proliferation in fetal hearts at E12.5 and E14.5, that are important stages of myocardium growth. The offspring of mice with pregestational diabetes had a significantly lower number of myocardial proliferating cells at E12.5 and E14.5 ($P < 0.05$, Figure 2.6 A-C). NAC treatment during gestation showed a trend but not a significant difference in cell proliferation at E12.5. Notably, this effect was significant at E14.5 ($P < 0.001$, Figure 2.6 C). In E12.5 endocardial cushion, cell proliferation was lower in the offspring of diabetic mice (Figure 2.7 A, B). NAC treatment resulted in significantly higher cell proliferation in endocardial cushion of control and diabetic mice (Figure 2.7 B). We also assessed cell apoptosis through immunohistochemical analysis of cleaved caspase-3 (Figure 2.7 A). Apoptosis in the endocardial cushion at E12.5 was significantly higher in the offspring of diabetic mice compared to controls ($P < 0.05$, Figure 2.7 A, C). NAC treatment had no significant effect on apoptosis in the offspring of diabetic mice, but increased apoptosis in the endocardial cushion in controls ($P < 0.05$, Figure 2.7 C). As a result of lower cell proliferation, myocardial wall thickness was lower in the offspring of diabetic mice at P0 ($P < 0.01$, Figure 2.8 A-C), which was rescued by NAC treatment ($P < 0.05$, Figure 2.8 A-C).
Figure 2.6. Cell proliferation in the fetal ventricular myocardium.

(A) Representative histological heart sections immunostained for pH3. pH3$^+$ staining (brown) is localized in the nucleus. (B and C) Quantification of pH3$^+$ cells in heart tissue sections, which include the right, left and septal myocardium. Data are means ± SEM, $n = 6$–8 per group $^*P < 0.05$ vs. untreated control, $^\dagger P < 0.001$ vs. untreated diabetes. Scale bar is 50 µm.
Figure 2.7. Cell proliferation and apoptosis in the endocardial cushion at E12.5

(A) Representative immunohistological staining of pH3 (nucleus) and cleaved caspase-3. (B and C) Quantification of pH3+ cells (cell proliferation) and cleaved caspase-3+ cells (apoptosis) in relation to total endocardial cushion cells. *P < 0.05 vs. untreated control, †P < 0.05 vs. untreated diabetes, **P < 0.001 vs. untreated control. Scale bar is 50 µm. N=6-7 hearts per group.
Figure 2.8. Myocardial wall thickness at P0.

(A) Representative images of myocardial wall thickness. (B and C) Quantification of right and left ventricular wall thickness. n = 5-6 per group, *P < 0.01 vs. untreated control, †P < 0.05 vs. untreated diabetes. Scale bar is 200 µm.
2.4.5 Effects of NAC on Transcription Factor Expression in Fetal Hearts from Diabetic Mice

Pregestational diabetes alters gene expression levels in the developing heart [29]. To study the effect of pregestational diabetes and NAC treatment on genes essential for early heart development, quantitative RT-PCR analysis was performed at E11.5. Our data showed transcript levels of Gata4 and Gata5 were lower in the embryonic hearts of diabetic mice at E11.5 ($P < 0.05$, Figure 2.8 A-B). Since GATA4 and GATA5 regulate cell proliferation in the fetal heart, we evaluated expression levels of cyclin D1, an important cell cycle regulator. Our data showed that pregestational diabetes significantly decreased cyclin D1 mRNA levels in the embryonic heart ($P < 0.01$). On the contrary, Vegf-a mRNA levels were increased in the embryonic hearts from diabetic mice ($P < 0.05$, Figure 2.9 C). These changes were all restored to control levels after NAC treatment ($P < 0.05$, Figure 2.9 A-D). However, other cardiac transcription factors including Nkx2.5, Mef2c and Tbx5 were not significantly altered by maternal diabetes or NAC treatment (Figure 2.9 E-G). In addition, levels of Bmp4 and Tgf-β1, which regulates cardiac valve formation, were not significantly altered (Figure 2.9 H-I).
Figure 2.9. Gene expression levels in fetal hearts at E11.5.

(A-D) The mRNA levels of Gata4, Gata5, Vegf-a and cyclin D1 were significantly altered in diabetic fetal hearts, which were restored to normal levels by NAC. (E-I) Neither pregestational maternal diabetes nor NAC treatment affected the mRNA levels of Tbx5, Nkx2.5, Tgf-β1, Mef2c and Bmp4 in the embryonic hearts by two way ANOVA. Data are means ± SEM, n = 6-8 hearts per group. *P < 0.05 vs. untreated control, †P < 0.05 vs. untreated diabetes.


2.5 DISCUSSION

Pregestational diabetes is a major risk factor for CHDs in humans. However, the molecular mechanisms that lead to the development of CHDs and possible therapeutic approaches to prevent those defects are still not fully understood. It is generally believed that oxidative stress plays a major role in the induction of birth defects in diabetic fetus [12, 16, 30]. Here, we employed a mouse model of pregestational diabetes induced by STZ and studied the effects of NAC treatment on CHDs in the offspring of diabetic mice. Our data showed that pregestational diabetes resulted in a high incidence of CHDs and decreased cell proliferation associated with altered expression levels of \textit{Gata4}, \textit{Gata5} and \textit{Vegf-a}. Importantly, GSH levels were decreased while ROS levels were increased in the fetal heart of pregestational diabetes. Notably, these abnormalities in the fetal heart were rescued by maternal treatment with NAC. Our study provides new evidence on the critical role of glutathione in embryonic heart development and suggests that NAC may have therapeutic potential in preventing CHDs in patients with pregestational diabetes.

To simulate congenital malformations induced by maternal diabetes without genetic modifications, several experimental approaches have been used, which include STZ- or alloxan-induced diabetes, and infusion of glucose to induce hyperglycemia. When diabetes or hyperglycemia is induced at the time of mating or within a few days after gestation, congenital defects in the central nervous system and skeletal malformation are observed in the offspring [31, 32]. While congenital heart malformations have been observed in animal studies of diabetic pregnancy, a spectrum of defects that can arise has been less well characterized [33-35]. In the present study, diabetes was induced by STZ in female mice for at least one week before gestation to
avoid the potential teratogenic effects of STZ on developing embryo. Our results show that pregestational diabetes induces embryopathy with a wide range of cardiovascular malformations including ASD, VSD, AVSD, TGA, DORV and TOF. These malformations of the cardiovascular system mirror congenital defects of neonates born to females with pregestational diabetes [6]. Thus, our model represents an appropriate animal model to study CHDs induced by pregestational diabetes.

Diabetes increases ROS production through increased activity of ROS generating enzymes and decreased activity of antioxidant enzymes [36, 37]. Extensive evidence have shown the involvement of oxidative stress in diabetic embryopathy [38] and the importance of glutathione in regulating ROS levels and redox signaling [26]. In the present study, we demonstrated that ROS levels were significantly higher in the fetal heart of diabetic offspring. Furthermore, total glutathione, GSH and GSSG levels were lower in the embryonic heart of diabetic offspring. To replenish GSH levels in the embryonic heart of diabetic mice, female mice with pregestational diabetes were treated with NAC, a precursor of cysteine essential for the production of GSH [18, 39, 40]. Notably, treatment with NAC normalized GSH levels and ROS levels in the fetal heart of diabetic mice. Importantly, NAC treatment also resulted in significantly fewer CHDs induced by pregestational diabetes. These data suggest an important role of GSH depletion and excessive ROS production in the development of CHDs. Previous studies have shown that treatment with NAC in vitro or GSH ethyl ester in vivo reduces gross embryonic malformation induced by high glucose or maternal diabetes [10, 16]. However, the beneficial effect of glutathione on cardiac development was limited to outflow tract defects induced by high glucose [20, 34]. The present study further
demonstrated the beneficial effects of NAC on a wide spectrum of cardiovascular malformations induced by pregestational diabetes in vivo. Additionally, NAC treatment reduced water and food intake of diabetic mice. While this phenomenon is also observed in NAC treated diabetic rats [41] our knowledge of the underlying mechanism is limited. NAC has been demonstrated to delay the onset of type 1 diabetes in biobreeding rats [42] and protect against hyperglycemia induced insulin resistance in rats [43]. Although, the beneficial effects of NAC in diabetic condition are explained by replenishing GSH levels and depleting oxidative stress [44], further studies are required to examine whether NAC has an effect on intracellular glucose metabolism or insulin sensitivity in diabetic condition. It should be noted that NAC treatment did not alter total glutathione levels in the fetal hearts of control mice. This is not surprising because intracellular GSH levels are regulated by a feedback inhibition to glutamate-cysteine ligase (GCL), a rate limiting enzyme in the production of GSH [45]. As such, the exogenous NAC participates in GSH synthesis only during oxidative stress conditions [39]. In addition, NAC also protects GSH from oxidation through its antioxidant properties independent of GSH synthesis [46], leading to an increased GSH/total glutathione ratio in both diabetic or control mice in the present study.

It is well documented that ROS regulates gene expression, cell proliferation and apoptosis [47]. In the present study, transcription factors that are critical to embryonic heart development including Gata4, Gata5 [48, 49] were downregulated in diabetic fetal hearts at E11.5. However similar to previous studies, Vegf-a mRNA levels were higher in diabetic fetal hearts [35]. Elevated expression levels of VEGF-A are associated with CHDs [50, 51]. High VEGF-A levels in fetal hearts inhibit epithelial-to-mesenchymal
transition (EMT) in the endocardial cushion, which contributes to formation of atrioventricular septum [52, 53]. Although a causal relationship between altered expression of *Gata4, Gata5, Vegf-a* and the development of CHDs in our study cannot be established, the fact that treatment with NAC restored their expression, improved cell proliferation via restoring cyclin D1 expression and prevented CHDs in diabetic offspring suggests that these transcription factors are sensitive to redox regulation and their alteration may contribute at least in part to cardiac malformation in pregestational diabetes.

Apoptosis is a highly regulated process and aberrant apoptosis may result in cardiovascular defects [54, 55]. The present study showed that apoptosis in the endocardial cushion was higher in diabetic embryos. Additionally, treatment with NAC resulted in higher apoptosis and induced 10% septal defects in control embryos. These data are consistent with a role of apoptosis in cardiac malformation [55, 56]. Cell proliferation was assessed using pHH3 staining, which marks cells undergoing mitosis. Notably, the number of pHH3⁺ cells in the endocardial cushion and myocardium was lower in diabetic embryos, which was rescued by NAC treatment. Cell proliferation in endocardial cushion was also higher with NAC treatment in the control embryos. Since NAC treatment did not inhibit apoptosis in diabetic embryos in our study, higher cell proliferation may represent a major effect of NAC in preventing CHDs in diabetes.

In conclusion, the present study demonstrated that pregestational diabetes in mice induces a wide spectrum of CHDs similar to humans. Treatment with NAC increases GSH levels, decreases ROS levels in the fetal heart and prevents the development of CHDs in the offspring of pregestational diabetes. In women with pregestational diabetes, insulin is the
primary treatment to achieve good glycemic control [57]. However, insulin treatment is not sufficient to decrease the risk of CHDs in the diabetic offspring to normal levels [38, 58]. Even with optimal care and planning of diabetic pregnancies, the risk of CHDs in the offspring of diabetic mothers is not as low as in the offspring of nondiabetic mothers (1-5%). Further studies are required to investigate whether NAC, an FDA approved drug either alone or in combination with insulin prevents CHDs in infants of women with pregestational diabetes.

2.6 REFERENCES


52. Hong JP, Li XM, Li MX, Zheng FL: VEGF suppresses epithelial-mesenchymal transition by inhibiting the expression of Smad3 and miR192, a Smad3-dependent microRNA. *Int J Mol Med* 2013, 31(6):1436-1442.


3. CHAPTER 3

Pregestational Diabetes Induces Fetal Coronary Artery Malformation via Reactive Oxygen Species Signalling

A version of this chapter has been submitted to *Diabetes*.

Hoda Moazzen, Xiangru Lu, Murong Liu, Qingping Feng.

**Pregestational Diabetes Induces Fetal Coronary Artery Malformation via Reactive Oxygen Species Signaling**

*Diabetes* 2014 acceptable with minor revisions
3.1 CHAPTER SUMMARY

Hypoplastic coronary artery disease is a congenital coronary artery malformation associated with a high risk of sudden cardiac death. However, the etiology and pathogenesis of hypoplastic coronary artery disease remain undefined. Pregestational diabetes increases reactive oxygen species (ROS) levels and the risk of congenital heart defects. We hypothesized, that pregestational diabetes induces coronary artery malformation via ROS signaling. Our data show that pregestational diabetes in mice induced by streptozotocin significantly increased ROS production and decreased coronary artery diameter and volume in fetal hearts. Pregestational diabetes also impaired epicardial epithelial-to-mesenchymal transition (EMT) as shown by analyses of the epicardium, epicardial derived cells and fate mapping. Additionally, ROS-dependent hypoxia inducing factor (Hif-1α) and epicardial EMT signaling pathway was examined. The expression of Hif-1α, Snail1, Slug, basic fibroblast growth factor (bFgf) and retinaldehyde dehydrogenase (Aldh1a2) were lower while E-cadherin expression was higher in the fetal heart of diabetic mothers. Notably, these abnormalities were all rescued by treatment with an antioxidant N-Acetylcysteine (NAC) in diabetic females during gestation. Ex vivo analysis shows that high glucose inhibited epicardial EMT, which was reversed by NAC treatment. We conclude that pregestational diabetes in mice can cause coronary artery malformation via ROS signaling. Our study may provide a rationale for further clinical studies to investigate whether pregestational diabetes could cause hypoplastic coronary artery disease in humans.
3.2 INTRODUCTION

Pregestational diabetes is a risk factor for CHDs in infants [1,2]. Clinical studies have shown that pregestational diabetes increases the risk of CHDs in the offspring by 3–5 fold compared to non-diabetic pregnancies [1-3]. To date, analysis of congenital heart malformation in the newborn is mainly restricted to major cardiac structures, which include aorta, pulmonary artery, atrioventricular septum, cardiac valves and the myocardium, but coronary arteries are not routinely examined [4]. Importantly, congenital malformation can occur in coronary arteries leading to null coronary artery or hypoplastic coronary artery disease [5]. While null coronary artery is embryonically lethal, hypoplastic coronary artery disease is a rare congenital coronary abnormality defined by malformation of one or more major branches of the coronary arteries with a marked decrease in luminal diameter and length. Hypoplastic coronary artery disease can be asymptomatic, but is often associated with myocardial infarction and sudden cardiac death during intense physical activities [6]. However, the etiology and pathogenesis of hypoplastic coronary artery disease remain undefined. Furthermore, it is not known if pregestational diabetes results in coronary artery malformation in the offspring. Isolated cases of congenital coronary artery abnormalities have been identified in infants by autopsy. However, whether the mothers of these patients had pregestational diabetes was not disclosed in these reports [7,8]. A large multicenter case-control study showed a significant association between pregestational diabetes and a wide range of CHDs [1]. Since coronary arteries were not routinely examined, defects of coronary arteries were not reported in this study. Notably, a recent population-based study reported cases of
coronary artery anomaly born from mothers with obesity. However, patients with established diabetes were excluded from this study [9].

Coronary arteries are developed from several sources of progenitors, which include endothelial cells of sinus venosus, proepicardial organ and the endocardium [10-12]. The current understanding is that cells from the proepicardial organ migrate toward the myocardium and form the epicardium. Concurrent with formation of the epicardium, some epicardial cells undergo epithelial-to-mesenchymal transition (EMT), migrate to the subepicardial space and myocardium and become epicardial derived cells (EPDCs). Simultaneously, a sub-population of endothelial cells of the sinus venosus also migrates to the subepicardial space, forming subepicardial endothelial progenitors [11]. When resident in the myocardium, the EPDCs give rise to vascular smooth muscle cells and fibroblasts [13] while subepicardial endothelial progenitors give rise to coronary endothelial cells [14]. Together, EPDCs and subepicardial endothelial cells contribute to the development of coronary arteries with a minor contribution from the endocardium.

Epicardial EMT is regulated by several epicardial transcription factors and myocardial induced signalling molecules [15]. An important factor in vasculogenesis and heart development is hypoxia inducible factor (HIF-1α) [16], which regulates many downstream target genes, including Wilms tumour-1 (Wt1), a critical transcription factor expressed in epicardial progenitors and EPDCs [17,18]. Studies have shown that Wt1 directs epicardial progenitors to become smooth muscle cells and fibroblasts, and form coronary arteries [19]. Pregestational diabetes increases reactive oxygen species (ROS) production [20,21] and downregulates HIF-1α expression in embryos [22]. We have shown that treatment with an antioxidant N-Acetylcysteine (NAC) increases reduced
glutathione (GSH) levels and decreases ROS production in the fetal hearts of diabetic offspring [20]. The present study was designed to test the hypothesis that pregestational diabetes impairs epicardial EMT and induces coronary artery malformation in the offspring. We further hypothesized that these abnormalities are mediated by ROS signaling and can be rescued by NAC treatment in diabetic females during gestation.

### 3.3 METHODS

#### 3.3.1 Animals

C57BL/6 wild type, Wt1creERT2/+ and Rosa26mTmG mice were purchased from Jackson Laboratory (Bar Harbor, Maine). Animals in this study were handled in accordance with the Guide for the Care and Use of Laboratory Animals, published by the U.S. National Institutes of Health (NIH publ. no. 85-23, revised 1996). All procedures involving mouse handling 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.

#### 3.3.2 Induction of Diabetes and NAC Treatment

Eight week old C57BL/6 female mice were induced to diabetes by streptozotocin (STZ, 80 mg/kg body weight per day, IP) injections for three consecutive days as described previously [20,23,24]. STZ was dissolved in sodium citrate (pH 4.0) and mice treated with sodium citrate were served as controls. Non-fasting blood glucose levels were determined one week after STZ injection using a glucose meter (OneTouch Ultra2; LifeScan Canada, Burnaby, BC). Mice with blood glucose levels higher than 11 mmol/l were considered diabetic and bred to normal adult males. Vaginal plugging was
monitored as a sign of successful mating and the day of vaginal plugging was recorded as day E0.5 of gestation. A subgroup of control and diabetic mice received 4 mg/ml N-Acetylcysteine (NAC, 1 g/kg body weight/day) in drinking water from E0.5 to the end of gestation or harvesting of the embryos [20].

3.3.3 Fate Mapping Analysis

Fate mapping of Wt1⁺ lineage was performed using \( Wt1^{creERT2/+} \) (Stock #10912, Jackson Laboratory) and the double fluorescent Cre reporter line \( Rosa26^{mTmG} \) (Stock #7576). \( Wt1^{creERT2/+} \) is a heterozygous \( Wt1^{-/+} \) mouse, where exon 1 of the \( Wt1 \) gene is deleted and replaced by a \( CreERT2 \) sequence under the control of the \( Wt1 \) promoter [19]. \( CreERT2 \) is created by fusion of Cre recombinase to a modified human estrogen receptor ligand binding domain (ERT2) (Figure 3.1). The \( Rosa26^{mTmG} \) reporter mice possess \( loxP \) sites on either side of a membrane-targeted tomato (mT) cassette followed by membrane-targeted green fluorescence protein (GFP, mG) cassette. In the absence of Cre recombinase activity, mT (red fluorescence) is expressed in all tissues. In the presence of Cre, the mT cassette is deleted and mG (green fluorescence) is expressed only in the targeted cells. Female \( Rosa26^{mTmG} \) mice were induced to diabetes by STZ (80 mg/kg body weight per day, IP for 3 days) as described above, and were bred with \( Wt1^{creERT2/+};Rosa26^{mTmG} \) males. Tamoxifen (50 mg/kg body weight, IP) was injected to diabetic or control pregnant females at E10.5. Upon tamoxifen administration, Cre is activated in Wt1 expressing cells and induces recombination to express GFP, which is one of the two fluorescence proteins in the \( Wt1^{creERT2/+};Rosa26^{mTmG} \) offspring. Embryos were harvested at E14.5. Double transgenic \( Wt1^{creERT2/+};Rosa26^{mTmG} \) embryos were sectioned and
immunostained for GFP. The Wt1\(^+\) derivatives, which are GFP\(^+\) cells and structures, were quantified and compared between diabetic and control mice.

**Figure 3.1. Lineage tracing to evaluate derivatives of epicardial Wt1\(^+\) cells.**

A) A schematic diagram of a breeding plan to generate offspring with Rosa\(^{mTmG}\), Wt1\(^{CreERT2}\) genotype. B) Red fluorescence protein (RFP) is transcribed in cells with no Wt1 expression. CreERT2 is transcribed in Wt1 expressing cells. Upon tamoxifen administration, Cre is expressed and induce recombination at LoxP sites, thus Wt1 expressing cells and their lineage will express green fluorescence protein (GFP).
3.3.4 Analysis of Superoxide Production

Embryonic heart tissues were harvested in all four groups at E14.5. Frozen samples were cut into 10-µm sections using a cryostat (CM1950, Leica). Superoxide levels were assessed by incubation of heart sections with 2 µM dihydroethidium (DHE) (Invitrogen Life Technologies) for 30 minutes in a humidified and light protected chamber at 37 °C [20]. DHE florescence signals were detected using a florescence microscope (Observer D1, Zeiss). At least 5 images were captured using fixed exposure time for each heart and the intensity of DHE florescence signals per myocardial area were quantified using AxioVision software (Zeiss, Germany).

3.3.5 Immunohistochemistry Analysis

Formation of coronary artery branches was studied at E16.5 and P0 when the coronary arteries are formed. Immunohistochemical analysis was performed to study formation of coronary vasculature and their precursors. In brief, the thoraxs of mice were harvested and processed in 4% paraformaldehyde overnight, dehydrated in ethanol, embedded in paraffin medium and sectioned transversely to 5 µm sections. Prior to immunostaining, antigen retrieval was performed in citric acid buffer (0.01 M, pH 6.0) for 12 minutes at 94 °C using a microwave oven (BP-111, Microwave Research & Applications, Carol Stream, Illinois). Samples were incubated with primary antibody overnight: anti-α-smooth muscle actin (1:3000, Sigma-Aldrich, Toronto, ON, Canada), biotinylated grifonia simplicifolia lectin-1 (1:250, Vector Laboratories, Burlington, ON, Canada), anti-Wilm’s tumor-1 (1:300, Calbiochem, Etobicoke, ON, Canada), anti-E-cadherin (1:200, Santa Cruz biotechnology, Santa Cruz, CA, USA), anti-GFP (1:1000, Abcam, Toronto, ON, Canada), anti-phospho-histone H3 (pHH3, 1:500, Abcam, Toronto,
ON, Canada) and anti-cleaved caspase-3 (1:800, Cell Signaling, Danvers, MA, USA) followed by one of the following secondary antibodies (Vector Laboratories, Burlington, ON, Canada) for an hour: biotinylated goat anti-rabbit IgG (1:500), biotinylated goat anti-mouse IgG (1:500) or biotinylated donkey anti-goat IgG (1:500). Signals were amplified by incubation with the ABC reagent (Vector Laboratories, Burlington, ON, Canada) and visualized using 3-3’di-aminobenzidine tetrahydrochloride (Sigma-Aldrich, Toronto, ON, Canada). Heart sections were counterstained with hematoxylin (Thermo Scientific, Waltham, MA USA) and images were captured using a light microscope (Observer D1, Zeiss, Germany). To visualize and analyze coronary artery volume, images of every 25 µm heart sections immunostained with α-smooth muscle actin were captured and imported to AMIRA® program for three-dimensional reconstructions [20]. Coronary arteries and the myocardium wall thickness were labeled individually. Using analytical tools of AMIRA, pixels were converted to volume (µl) and a ratio of coronary artery volume to myocardial volume was obtained.

3.3.6 Ex vivo Heart Explant Culture

Epicardial cells of E12.5 hearts undergo epithelial to mesenchymal transition (EMT) when they are cultured on collagen gel. To determine whether hyperglycemia impairs EMT, the ventricles of E12.5 embryos from control dams were harvested and cultured on collagen gel. Collagen (1 mg/ml, type I collagen of rat’s tail, BD Bioscience) was prepared in M199 media (M5017, Sigma), which contained 5 mM D-glucose or in M199 with addition of 25 mM D-glucose, which made the final D-glucose concentration to be 30 mM. Casted collagen was hydrated by OPTI-MEM media plus 1% fetal bovine serum (FBS) and insulin-transferrin-selenium (ITS) for 30 minutes at 37 °C. E12.5
ventricles were explanted and incubated at 37 °C overnight. The following day, 5 or 30 mM D-glucose in M199 media with 10% FBS were added to the heart explants. The glucose concentration was about 5 mM higher than the average blood glucose of diabetic dams at E10.5. To inhibit ROS production, heart explant cultures were treated with 0.5 mM NAC. The number of spindle shaped cell outgrowths from the explanted ventricles was quantified 4 days post culturing. Images were captured using phase contrast microscope (Zeiss, Germany).

### 3.3.7 Real-time RT-PCR Analysis

The epicardium is formed at E10.5 and EMT is initiated followed by formation of the epicardial cell layer. To examine transcript levels that are important for EMT we harvested hearts from E11.5 embryos. Total RNA was extracted from individual E11.5 embryonic hearts using RNeasy Mini kit (Qiagen, Burlington, ON, Canada) as per manufacturer’s instructions. cDNA was synthesized from 0.1 µg of total RNA using M-MLV reverse transcriptase in 10 µl reactions, which were diluted by 3 times for genes of interest and 500 times for a housekeeping gene 28S, respectively. Two microliters of diluted cDNA were used for real-time PCR amplification using EvaGreen qPCR MasterMix (Applied Biological Materials, Vancouver, BC, Canada). Specific primers were designed for Hif-1α, Snail1, Slug, bFgf, β-catenin and Aldh1a2 (Table 3.1). Samples were amplified for 35 cycles using Eppendorf Realplex (Eppendorf, Hamburg, Germany). The mRNA levels in relation to 28S ribosomal RNA were determined using a comparative C_T method [25].
3.3.8 Statistical Analysis

Data are presented as means ± SEM. Statistical analysis was performed using two-way analysis of variance (ANOVA) followed by Bonferroni post test (GraphPad Prism program, version 5.0). The incidence of malformation was assessed by Chi-square test. A $P$ value of less than 0.05 was considered statistically significant.
Table 3.1. Primer sequences for real-time PCR analysis

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<td>NM_011427.2</td>
<td>114</td>
<td>F: CACACGCTGCTTTGTCT</td>
</tr>
<tr>
<td></td>
<td></td>
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</tr>
<tr>
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<td>159</td>
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</tr>
<tr>
<td></td>
<td></td>
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<td>R: AGGTTGCGGGGTTGATAG</td>
</tr>
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</table>
3.4 RESULTS

3.4.1 Pregestational Diabetes Increases ROS Levels in Fetal Hearts

To assess ROS production, dihydroethidium (DHE) was employed as a probe to detect superoxide generation in the fetal heart. A significant increase in DHE fluorescence reading in the E14.5 hearts of the offspring of diabetic mice indicated excess superoxide levels (Figure 3.2 A-B). Notably, treatment with an antioxidant NAC in drinking water in the diabetic dams during gestation significantly inhibited superoxide generation in the fetal hearts of diabetic mice (Figure 3.2 A-B). These data suggest that pregestational diabetes increases ROS levels in the fetal heart, which are effectively inhibited by NAC treatment.

3.4.2 Coronary Artery Malformation in Fetal Hearts from Pregestational Diabetes

A critical period for coronary artery development is from E9.5 to E14.5 in mice. To examine the effect of pregestational diabetes on coronary artery formation, adult female mice were induced to diabetes by STZ and their non-fasting blood glucose levels were determined during gestation. Our data show that blood glucose levels in diabetic females were significantly higher than normal controls ($P<0.001$, Table 3.2). NAC treatment did not significantly alter blood glucose levels in the diabetic and normal dams (Table 3.2).

Formation of coronary artery and capillaries was evaluated following immunostaining of $\alpha$-smooth muscle actin and lectin-1, respectively. Offspring of diabetic mothers show lower left and right coronary artery diameter and abundance at P0.
Figure 3.2. Reactive oxygen species (ROS) production assessed by dihydroethidium (DHE) fluorescence in fetal hearts at E14.5

A) Representative images of DHE red fluorescence in the LV myocardium. B) Quantification of DHE fluorescence per myocardial area. N=5 fetal hearts from 2 to 3 litters per group. *P<0.05 vs. untreated control, †P<0.01 vs. untreated diabetes. Scale bar 20 μm.
Table 3.2. Non-fasting blood glucose levels (mM) during gestation in mice

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Diabetes</th>
<th>Control+NAC</th>
<th>Diabetes+NAC</th>
</tr>
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<tr>
<td>Pregnant mice (n)</td>
<td>8</td>
<td>10</td>
<td>8</td>
<td>10</td>
</tr>
<tr>
<td>At E0.5</td>
<td>6.9 ± 0.3</td>
<td>17.2 ± 1.6 *</td>
<td>7.5 ± 0.3</td>
<td>14.9 ± 1.0 *</td>
</tr>
<tr>
<td>At E10.5</td>
<td>8.6 ± 0.5</td>
<td>24.2 ± 2.2 *</td>
<td>8.9 ± 0.5</td>
<td>22.0 ± 1.4 *</td>
</tr>
</tbody>
</table>

Data are mean ± SEM. *P<0.05 vs. respective controls. Fetuses and neonates from these dams were used for histological, immunostaining, ROS and real-time PCR analysis, and ex vivo heart cultures. (Figure 3.3 A-B) and at E16.5 (Figure 3.4 A-B). Additionally, coronary arteries in offspring of diabetic mice had lower numbers of smooth muscle cells surrounding the vessels (Figure 3.3 C) and lower capillary abundance at P0 (Figure 3.3 D). Impaired coronary artery development is illustrated in three-dimensional reconstructions of coronary arteries, which demonstrate smaller arteries with less arborization in offspring of diabetic mice at E16.5 and P0 (Figure 3.5 A). The impaired coronary artery development was not simply due to changes in myocardial volume as the ratio of total coronary artery volume to myocardial volume was lower in the offspring of diabetic mice at P0 and E16.5 (Fig. 3.5 B-C). The incidence of coronary artery malformation was 46% (see Table 3.3). To study the role of ROS in coronary artery malformation, diabetic and control dams were treated with an antioxidant NAC in their drinking water during
Figure 3.3. Malformation of coronary vasculature in the offspring of diabetic mice restored by NAC treatment at P0.

Smooth muscle and endothelial cells were marked by α-smooth muscle actin (A-C) and lectin-1 (D) immunostaining (brown), respectively. A: Representative images of the left main coronary artery branch at the aortic root. B: Representative images of coronary artery branches in the myocardium. Arrows are pointed to coronary vessels. C: Coronary artery vessels in the myocardium. D: Capillaries in the myocardium. E and F: Diameters of the left and right coronary arteries at the aortic root (n=12-15 hearts per group). G: Coronary artery abundance per mm² myocardium (n=7-10 hearts per group). H: Capillary density per mm² myocardium (n=5 hearts per group). Data were collected from 3 to 5 litters per group. *P<0.01 vs. untreated control, †P<0.01 vs. untreated diabetes. AO, aorta; RV, right ventricle; LV, left ventricle; NAC, N-Acetylcysteine. Scale bars are 50 µm in A, C, D and 400 µm in B.
Figure 3.4. Malformation of coronary artery vasculature in the fetal heart of diabetic mice restored by NAC treatment at E16.5

A) Representative image of left coronary artery at the aortic root. B) Representative image of coronary artery branches in the ventricular myocardium. C) Panel C is zoom-up images of the boxed areas in B to demonstrate differences in vessel size. D) and E) Quantification of left and right coronary artery diameter. F) Quantification of coronary artery abundance. N=7-8 samples per group. *P<0.05 vs. untreated control, †P<0.01 vs. untreated diabetes.
Figure 3.5. Analysis of three-dimensional (3D) reconstructions of coronary artery tree demonstrates reduced coronary artery volume in the offspring of diabetic mice is restored by NAC treatment at E16.5 and P0

A) Illustrations of 3D reconstructions of coronary arteries. B and C) Quantification of the total coronary artery volume normalized to myocardial volume at E16.5 and P0, respectively. N=5 hearts from 3 litters per group. * P<0.05 vs. untreated control, †P<0.01 vs. untreated diabetes. RV, right ventricle; LV, left ventricle; NAC, N-Acetylcysteine.
gestation. Notably, abnormalities in coronary artery and capillary development in the diabetic offspring were rescued by NAC treatment (Figure 3.3, Figure 3.4, and Figure 3.5). NAC treatment also significantly rescued the incidence of coronary artery malformation in the offspring of diabetic mice to 6% \((P<0.01, \text{Table 3.3})\).

### 3.4.3 Pregestational Diabetes Impairs Epicardial EMT in Fetal Hearts

To gain a better understanding of cellular and molecular events that caused coronary artery malformation in the offspring of diabetic mice, proepicardial organ and the epicardium were studied during embryogenesis using presence of Wt1 as a marker. Our data show that pregestational diabetes did not alter the number of Wt1\(^{+}\) cells in the proepicardial organ at E9.5 (Figure 3.6). However, the epicardium in the embryonic heart of diabetic mice formed a sparse and loosely attached cell layer to the myocardium with significant reductions in the number of Wt1\(^{+}\) epicardial and subepicardial cells at E12.5 (Figure 3.7 A, D and E). Impaired Wt1 expression in fetal hearts of diabetic mothers was persistent at E14.5, as evidenced by a lower number of Wt1\(^{+}\) cells in the epicardial cell layer and in the compact myocardium (Figure 3.7 B, F and G). Concurrent with lower in the number of epicardial Wt1\(^{+}\) cells, the number of E-cadherin positive epicardial cells was higher in fetal hearts of diabetic mothers at E12.5 (Figure 3.7 C, H). Notably, NAC treatment during gestation reduced epicardial E-cadherin expression and restored the number of Wt1\(^{+}\) cells in the epicardium, subepicardium and myocardium in fetal hearts (Figure 3.7 A-H).

To directly evaluate the derivatives of epicardial Wt1\(^{+}\) cells, fate mapping analysis was performed using Rosa\(^{mTmG}\) female mice, which were induced to have diabetes by STZ and then bred to tamoxifen inducible Wt1\(^{\text{CreERT2/++;Rosa\(^{mTmG}\)}}\) males. Following
tamoxifen treatment in the female mice at E10.5, Wt1\(^+\) derivatives including coronary vessels were observed in the hearts of Wt1\(^{\text{CreERT2/++;Rosa}^\text{mTmG}}\) embryos at E14.5 (Figure 3.7 I). In agreement with our hypothesis, pregestational diabetes diminished Wt1\(^+\) lineage and coronary vessels in the hearts of Wt1\(^{\text{CreERT2/+;Rosa}^\text{mTmG}}\) embryos (Figure 3.7 I-K).

**Table 3.3. Incidence of coronary artery malformation (CAM) and congenital heart defects (CHDs) in fetuses from E16.5 to E18.5**

<table>
<thead>
<tr>
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<th>Control</th>
<th>Diabetes</th>
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<tr>
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<tr>
<td>CAM/total fetuses (n/N)</td>
<td>0/26</td>
<td>13/28*</td>
<td>0/25</td>
<td>2/34 †</td>
</tr>
<tr>
<td>CHDs/total fetuses (n/N)</td>
<td>0/26</td>
<td>17/28*</td>
<td>0/25</td>
<td>2/34 †</td>
</tr>
<tr>
<td>CHDs+CAM/total fetuses (n/N)</td>
<td>0/26</td>
<td>11/28*</td>
<td>0/25</td>
<td>1/34 †</td>
</tr>
<tr>
<td>CAM (%)</td>
<td>0</td>
<td>46</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>CHDs (%)</td>
<td>0</td>
<td>61</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>CHDs+CAM (%)</td>
<td>0</td>
<td>39</td>
<td>0</td>
<td>3</td>
</tr>
</tbody>
</table>

CHDs include ventricular septal defects, atrioventricular septal defects, transposition of great arteries, and double outlet right ventricle. *\(P<0.001\) vs. control; †\(P<0.001\) vs. diabetes by Chi-square analysis.
Figure 3.6. Wt1\(^+\) cells in the proepicardial organ (PEO) at E9.5.

A) Representative images of PEO of control and diabetic offspring. B) Quantification of Wt1\(^+\) cells to total number of cells in the PEO. N=6 samples per group. There was no statistical significance in the number of Wt1\(^+\) cells in the PEO between control and diabetic offspring.
Figure 3.7. Analysis of the epicardium and fate mapping of Wt1\(^+\) lineage in hearts of diabetic offspring.

A and B) Representative heart sections of Wt1 immunostaining at E12.5 and E14.5, respectively. Arrows point to the epicardium in A and arrow heads point to Wt1\(^+\) cells in the subepicardium and myocardium in A & B, respectively. C) Representative heart sections of E-cadherin immunostaining at E12.5. Arrow heads indicate E-cadherin\(^+\) epicardial cells. D and E) Quantification of Wt1\(^+\) epicardial and subepicardial cell numbers, respectively. F and G) Quantification of Wt1\(^+\) cells in the epicardium and myocardium, respectively. H) Quantification of E-cadherin\(^+\) cells to total epicardial cell ratio (%). I-K: Fate mapping of Wt1\(^+\) lineage. I) Representative images of GFP staining in Wt1\(^{creERT2/+}\);Rosa\(^{mTmG}\) heart sections of control and diabetic offspring at E14.5. Brown color marks cells and vessels derived from Wt1\(^+\) lineage. J and K) Quantification of cells and vessels derived from Wt1\(^+\) lineage in the myocardium, respectively. N=5-6 hearts from 3 litters per group. *\(P<0.01\) vs. control or untreated control, †\(P<0.01\) vs. diabetes or untreated diabetes. Scale bars: 30 \(\mu m\).
3.4.4 ROS-dependent Downregulation of HIF-1α Signaling in Diabetic Fetal Hearts

To further understand ROS signalling in epicardial EMT, the expression of Hif-1α, a master regulator of vasculogenesis and epicardial EMT [26], and its downstream signalling molecules essential for coronary development were evaluated. The expression of Hif-1α was significantly lower in E11.5 fetal hearts of diabetic dams (Figure 3.8 A). Additionally, mRNA levels of Snail1 and Slug, which are key regulators of EMT, were significantly lower in fetal hearts of diabetic dams (Figure 3.8 B and C). Furthermore, retinoic acid signaling may be impaired in the fetal hearts of diabetic dams as evidenced by lower mRNA levels of Aldh1a2, which encodes the rate-limiting retinoic acid synthesis enzyme, retinaldehyde dehydrogenase 2 (RALDH2) [27], and its downstream target bFGF (Figure 3.8 D and E). Notably, these differences in the expression of Hif-1α, Snail1, Slug, Aldh1a2, and bFGF were restored in the hearts of offspring of diabetic females by NAC treatment (Figure 3.8 A-E). However, β-catenin mRNA levels that are essential for coronary artery formation [50] were not significantly altered by pregestational diabetes or by NAC treatment (Figure 3.8 F).

3.4.5 High Glucose Impairs Epicardial EMT ex vivo

To investigate whether hyperglycemia impairs epicardial EMT in fetal hearts of diabetic mothers, E12.5 hearts were cultured on collagen gel in both high glucose (30 mM) and normal glucose (5 mM) conditions for 4 days (Figure 3.9 A). The number of spindle shaped cells, which are epicardial cells, which have undergone EMT was quantified. Our data show that the number of spindle shaped cells was significantly lower
in high glucose compared to normal glucose cultures. The response was restored by NAC (0.5 mM) treatment (Fig. 3.9 A and B).

3.4.6 Pregestational Diabetes Diminishes Epicardial Cell Proliferation in Fetal Hearts

To assess the effect of pregestational diabetes on epicardial cell proliferation, immunostaining for pHH3, a marker of mitotic cells, was performed in fetal hearts. Our results show that the number of pHH3+ epicardial cells was significantly lower in fetal hearts of diabetic dams at E12.5 and E14.5 (Figure 3.10 A-D). Interestingly, treatment with NAC in diabetic dams completely restored epicardial cell proliferation at both time points (Figure 3.10 C and D). Assessment of the apoptotic epicardial cells in E12.5 hearts using immunostaining for cleaved caspase-3 show no significant difference between the control (0.37±0.16%) and diabetic groups (0.33±0.12%, n=8-10 hearts per group).
Figure 3.8. Gene expression of HIF-1α and its downstream targets critical to epicardial EMT in E11.5 hearts

mRNA levels were analyzed by real-time PCR. A) HIF-1α. B) Snail1. C) Slug. D) Aldh1a2. E) bFGF. F) β-Catenin. *P<0.05, **P<0.01 vs. untreated control, †P<0.05, ††P<0.01 vs. untreated diabetes. N=7-9 hearts from 3 to 4 litters per group.
Figure 3.9. High glucose inhibits EMT *ex vivo*.

A) Representative images of *ex vivo* E12.5 heart explant culture. Dashed line shows the border of migrated cells. B) Quantification of the number of spindle shaped cells, which are cells that have undergone EMT. Scale bar: 50 µm. *P<0.01 vs. untreated control (5 mM D-glucose), †P<0.01 vs. untreated high glucose (30 mM). N=10-12 hearts per group from 4 litters.
Figure 3.10. Reduced rate of epicardial cell proliferation in the fetal heart of diabetic mice restored by NAC treatment at E12.5 and E14.5.

A and B) Representative images of immunostaining of phospho-histone H3 (pHH3, brown) in E12.5 and E14.5 hearts, respectively. C and D) Quantification of pHH3+ epicardial cells per millimeter epicardium. N=6-8 hearts from 3 litters per group. Scale bar: 20 µm. *P<0.05 vs. untreated control, †P<0.01 vs. untreated diabetes.
3.5 DISCUSSION

To our knowledge, the present study demonstrated for the first time that pregestational diabetes impairs development of coronary artery vasculature in fetal hearts in an animal model. We further demonstrated that pregestational diabetes increases ROS production, diminishes HIF-1α and Wt1 expression, and decreases Snail/Slug and RALDH2/bFGF signalling pathways leading to disruption of epicardial EMT and malformation of fetal coronary arteries (for a schematic diagram of the proposed pathway, see Figure 3.11). Notably, inhibition of ROS production by antioxidant NAC restores epicardial EMT and prevents malformation of coronary arteries in the fetal heart of pregestational diabetes. Our study suggests a critical role of ROS signalling in coronary artery malformation during pregestational diabetes.

The embryonic epicardium is a major contributor to coronary artery development [28,29]. In this regard, cells from the epicardium undergo EMT and become EPDCs, which then differentiate into vascular smooth muscle cells and cardiac fibroblasts, leading to formation of coronary vessels. To study if pregestational diabetes affects epicardial formation, immunohistochemical analysis was performed. Although there were no significant changes in proepicardial organ progenitor cell numbers, the number of epicardial cells was lower and the epicardium was detached from the myocardium with lower in epicardial cell proliferation at E12.5 and E14.5 in embryonic hearts of diabetic mothers. Epicardial attachment to the myocardium is critical to epicardial cell proliferation and formation of the epicardium [30]. Our study shows that pregestational diabetes inhibits epicardial development likely via an interruption of epicardial attachment to the myocardium and a reduction of epicardial cell proliferation. The number of epicardial cells
Pregestational diabetes increases ROS via hyperglycemia and hypoxia. ROS production inhibits HIF-1α and Wt1 expression in the fetal heart. Downregulation of Wt1 decreases epicardial EMT and results in coronary artery malformation via inhibition of Snail/Slug and RALDH2/bFGF pathways. These abnormalities were all prevented by treatment with an antioxidant NAC in the diabetic dams.

Figure 3.11. Schematic summary of ROS signaling on coronary artery malformation in the offspring of pregestational diabetes
undergoing apoptosis was low (0.3%) and no significant difference was observed at E12.5 between control and diabetic offspring. However, whether pregestational diabetes increases epicardial cell apoptosis in the offspring at other stages of development remains to be determined.

Epicardial EMT is a critical process in coronary artery development [31]. In the present study, we demonstrated that pregestational diabetes decreases epicardial EMT. This is supported by the following experimental data. First of all, the number of Wt1\(^+\) cells, which are EPDCs from epicardial EMT, was lower in the subepicardial space and in the compact myocardium of diabetic offspring. Secondly, high glucose inhibited epicardial cell outgrowth in cultured heart explants, and thirdly, fate mapping analysis revealed significantly lower Wt1\(^+\) cell lineage in the fetal heart of pregestational diabetes. The Wt1\(^{creERT2}\) line has been shown to label the epicardium and its derivatives [32]. The lower Wt1\(^+\) lineage in the fetal heart of diabetic offspring is consistent with our hypothesis that pregestational diabetes impairs epicardial EMT. To our knowledge, this is the first experimental evidence to show that pregestational diabetes inhibits epicardial EMT in the fetal heart.

HIF-1\(\alpha\) is a transcription factor that promotes vasculogenesis during embryonic development [33]. To this end, HIF-1\(\alpha\) signalling has been shown to regulate epicardial EMT and EPDC migration into the myocardium, both of which are critical in patterning the coronary vasculature during early cardiac vasculogenesis [26]. The effects of HIF-1\(\alpha\) are mediated by the expression of factors essential for coronary artery development including Wt1 [17,34]. Importantly, HIF-1\(\alpha\) is also ROS sensitive. While low levels of ROS increase HIF-1\(\alpha\) expression and promote cardiovascular differentiation, high levels
of ROS may inhibit HIF-1α activity by inhibiting the binding of co-activator p300 to HIF-1α [35,36]. Furthermore, diabetes via hyperglycemia, increases ROS levels and decreases the expression and activity of HIF-1α [37,38]. In the present study, ROS levels were significantly higher while HIF-1α expression and the number of Wt1+ epicardial cells and EPDCs were lower in the embryonic heart of pregestational diabetes. Notably, these changes were abrogated by NAC treatment, which reduces ROS levels in the embryonic heart. Our data show that pregestational diabetes impairs HIF-1α/Wt1 signalling pathway via elevated ROS levels in the fetal heart (Figure 3.11).

Wt1 has been shown to regulate epicardial EMT through the expression of its downstream targets of Snail1, Slug and RALDH2 [39-41]. In line with our observation of lower epicardial EMT, we further demonstrated that the expression of Snail1 and Slug was lower in the embryonic hearts of pregestational diabetes. Retinoic acid feeds into an essential signalling pathway crucial for epicardial formation, epicardial attachment to the myocardium, myocardial growth and proliferation, and coronary artery development [42,43]. bFGF is an important mediator in retinoic acid signalling that promotes epicardial EMT and vasculogenesis in the embryonic heart. Our results show that the expression levels of Aldh12a, which encodes RALDH2, and its downstream target bFGF were lower, suggesting an impaired retinoic acid signalling in the fetal heart of diabetic mothers. Thus, pregestational diabetes impairs both Wt1/Snail/Slug and Wt1/RALHD2/bFGF signalling pathways, which may contribute to decreased epicardial EMT and malformation of coronary arteries in the fetal heart (Figure 3.11).

Physiological levels of ROS signalling regulates vasculogenesis [44]. While basal endogenous ROS levels are critical to normal vascular development, excessive ROS
production during embryogenesis may inhibit vasculogenesis. In this regard, elevated ROS levels induce vasculopathy in the yolk sac of embryos of diabetic rats [45]. NAC is a precursor of cysteine and decreases ROS levels via increases in glutathione synthesis and antioxidant capacity [46]. The present study showed that ROS production was higher in the fetal heart of pregestational diabetes. Notably, NAC treatment in diabetic females during gestation diminished ROS levels and restored expression of critical factors essential for epicardial growth and EMT. Importantly, NAC treatment rescued coronary artery malformation induced by pregestational diabetes. Our study suggests a critical role of elevated ROS and its signalling in mediating coronary artery malformation in the offspring of pregestational diabetic mice. Previous studies have shown that hyperglycemia or pregestational diabetes induces CHDs, which can be prevented by antioxidant treatments (Chapter 2), [47-49]. The results are consistent with our recent findings that elevated ROS levels contribute to the development of CHDs in the offspring of mice with pregestational diabetes, which are rescued by NAC treatment (Chapter 2). Thus, NAC may have therapeutic potential in the prevention of congenital heart defects including coronary artery malformation in patients with pregestational diabetes.

In conclusion, the present study demonstrates that pregestational diabetes impairs epicardial EMT and coronary artery development in mice. These abnormalities are associated with increased ROS production and decreased HIF-1α/Wt1 signalling (Figure 3.11). Notably, these abnormalities were all prevented by an antioxidant NAC treatment, suggesting a key role of ROS signalling in the malformation of coronary arteries in pregestational diabetes. The coronary artery phenotype observed in the present study bears resemblance to human hypoplastic coronary artery disease, which has a high risk of
spontaneous myocardial infarction and sudden cardiac death. Our study suggests that pregestational diabetes could cause hypoplastic coronary arteries in mice. However, further clinical studies are required to determine whether pregestational diabetes increases the incidence of hypoplastic coronary artery disease in humans.

3.6 REFERENCES


4. CHAPTER 4

NADPH Oxidase NOX2 Is Required for Endocardial to Mesenchymal Transition and Heart Development

Moazzen H, Wu Y, Lu X, Aulakh S, Feng Q.

In preparation for submission.
4.1 CHAPTER SUMMARY

NADPH oxidases (NOX) are a major source of reactive oxygen species (ROS) production in the heart. ROS signalling regulates gene expression, cell proliferation, apoptosis and migration. However, the role of NOX in embryonic heart development remains elusive. We hypothesized that deficiency of Nox2 disrupts endocardial to mesenchymal transition (EndMT) and results in congenital septal and valvular defects. Our results show that 34% of Nox2<sup>−/−</sup> neonates had various congenital heart defects (CHDs) including atrial septal defects (ASD), ventricular septal defects (VSD), atrioventricular canal defects (AVCD), and malformation of atrioventricular (AV) and aortic valves. Notably, Nox2<sup>−/−</sup> embryonic hearts show abnormal development of the endocardial cushion as evidenced by reduced rate of cell proliferation and an increased rate of apoptosis. Additionally, lack of Nox2 disrupted EndMT of cushion explants <em>ex vivo</em>. Furthermore, treatment with N-Acetylcysteine (NAC) to reduce ROS levels significantly decreased the number of cells undergoing EndMT in wild type and Nox2<sup>−/−</sup> embryos. Importantly, deficiency of Nox2 reduced cardiac expression of Gata4, Tgß2, Bmp2, Bmp4 and Snail1, which are essential for EndMT and endocardial cushion development. We conclude that NOX2 is critical to EndMT and embryonic development of endocardial cushion-derived structures including heart valves and cardiac septum.
4.2 INTRODUCTION

Congenital heart defects (CHDs) are the most common birth defects in infants affecting 1-5% of live births [1]. Malformation of septal and valvular structures are reported in about 40% of patients with CHDs [2]. Cardiac morphogenesis is regulated by an intricate network of signalling molecules and transcription factors [3]. Advances in genetic analysis have assisted in identifying genomic factors responsible for morphological abnormalities in patients with CHDs [4]. Currently, about 20% of CHDs are attributed to chromosomal abnormalities, single gene mutations or environmental teratogens. However, the etiology of 80% of CHDs remains unknown [5], indicating our limited knowledge on factors that regulate cardiac morphogenesis.

Reactive oxygen species (ROS) are an important signalling molecules that modulate intracellular redox state and gene expression profiles to regulate cell proliferation, differentiation, apoptosis and migration [6,7]. An imbalance in ROS production may have adverse effects on fetal development [8,9]. To this end, we (Chapter 2) and others have shown that excessive ROS generation in the embryonic heart alters gene expression profile and induces a wide range of CHDs [10], suggesting a pivotal role of ROS production in normal heart development.

A critical process in embryonic heart development is the epithelial to mesenchymal transition (EMT). The endocardial EMT (EndMT) is initiated at E9.5 in mice when endocardial cushion swellings are formed in the outflow tract (OFT) and AV canal regions [11]. With contributions from neural crest cells, endocardial cushions at OFT form the aorticopulmonary septum and semilunar valves, while endocardial cushion at AV canal participate in the formation of AV valves and cardiac septum. EndMT is
regulated by transcription factors and signalling molecules produced in the adjacent myocardium and endocardium cushions [12].

NADPH oxidases (NOX) are a family of \( \text{O}_2^- \) and \( \text{H}_2\text{O}_2 \) producing enzymes expressed in both phagocytic and non-phagocytic cells [13]. The enzyme complex is composed of NOX proteins, \( \text{p}22^{\text{phox}} \), \( \text{p}40^{\text{phox}} \), \( \text{p}47^{\text{phox}} \), \( \text{p}67^{\text{phox}} \) and Rac GTPase. ROS production from NOX enzymes by phagocytes plays an important role in killing invading pathogens. The heart also expresses NOX proteins. A major source of intercellular ROS production in adult cardiomyocytes and embryonic cardiac stem cells is NOX2 and NOX4 [14,15]. Mutations of NOX genes result in chronic granulomatous disease (CGD), a rare disease occurring in 1:200,000-450,000 live births [16]. Notably, atrial septal defects are also seen in patients with CGD [17], suggesting that mutations of NOX genes or a lack of NOX-derived ROS production may cause CHDs. However, mechanisms by which NOX enzymes regulate heart development are not clear. In this study, we hypothesized that deficiency of Nox2 disrupts EndMT and results in congenital septal and valvular defects. To test this hypothesis, a Nox2 deficient mouse was employed. Our data show that Nox2\(^{-/-}\) mice have cardiac septal defects and valvular abnormalities. Furthermore, deficiency of Nox2 impairs EndMT and endocardial cushion development. Our study supports a critical role of Nox2-derived ROS signaling in promoting EndMT and embryonic heart development.
4.3 METHODS

4.3.1 Animals

Nox2<sup>-/-</sup> and C57Bl/6 mice were purchased from Jackson Laboratory (Bar Harbor, Maine). Genotype was confirmed by PCR analysis using the following primers: 5' AAGAGAAACTCCTCTGCTGTGAA 3' and 5' GTTCTAATTCCATCAG AAGCTTATCG 3', provided by Jackson Laboratory. A breeding program was implemented to harvest fetal and postnatal mice. Animals in this study were handled in accordance with the *Guide for the Care and Use of Laboratory Animals*, published by the U.S. National Institutes of Health (NIH publ. no. 85-23, revised 1996). All procedures involving mouse handling 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 Immunohistochemistry Analysis

Heart morphology was analyzed in postnatal day 0 (P0) mice. Briefly, the mouse thorax was fixed in 4% paraformaldehyde overnight, dehydrated in ethanol, embedded in paraffin medium and sectioned transversely to 5 µm sections. Prior to immunostaining, antigen retrieval was performed in citric acid buffer (0.01 M, pH 6.0) for 12 minutes at 94 °C using a microwave oven (BP-111, Microwave Research & Applications, Carol Stream, Illinois). Samples were incubated with primary antibodies overnight: anti-Ki67 (1:500, Abcam, Toronto, ON, Canada), anti-caspase-3 (1:800, Cell Signaling, Danvers, MA, USA), anti-Snail1 (1:300, Abcam, Toronto, ON, Canada), or anti-NOX2 antibody (1:500, BD transduction laboratories, Mississauga, ON, Canada) followed by one of the
following secondary antibodies (Vector Laboratories, Burlington, ON, Canada) for an hour: biotinylated goat anti-rabbit IgG (1:500) or biotinylated goat anti-mouse IgG (1:500). Signals were amplified by incubation with the ABC reagent (Vector Laboratories, Burlington, ON, Canada) and visualized using 3-3’di-aminobenzidin tetrahydrochloride (Sigma-Aldrich, Toronto, ON, Canada). Heart sections were counterstained with hematoxylin (Thermo Scientific, Waltham, MA USA) and images were captured using a light microscope (Observer D1, Zeiss, Germany). Endocardial cushion cell density was calculated at E10.5 by quantifying the number of cells in endocardial cushion per area.

4.3.3 Analysis of Superoxide Levels

Embryonic heart tissues were harvested at E10.5. Frozen samples were cut into 10-µm sections using a cryostat (CM1950, Leica, Germany). Superoxide levels were assessed by incubation of heart sections with 2 µM dihydroethidium (DHE, Invitrogen Life Technologies, Burlington, Canada) for 30 minutes in a humidified and light protected chamber in room air at 37°C [18]. DHE fluorescence signals were detected using a fluorescence microscope (Observer D1, Zeiss, Germany). For analysis of superoxide levels, 5 images of each heart sample were captured using fixed exposure time for both groups. The intensity of fluorescence signals per myocardial area was quantified using AxioVision software. A limitation of this assay is that the oxygen level was not adjusted to that of the embryonic hearts in vivo [19].
4.3.4 Real-time RT-PCR Analysis

Total RNA was extracted from E10.5 fetal hearts using RNeasy Mini kit (Qiagen, Burlington, ON) as per manufacturer’s instructions. cDNA (100 ng) was synthesized using M-MLV reverse transcriptase. Real-time PCR was conducted using EvaGreen qPCR MasterMix (Applied Biological Materials, Vancouver, BC). Specific primers were designed for Nkx2.5, Gata4, Gata5, Tbx5, Bmp2, Bmp4, Tgf-β1, Tgf-β2, Notch1, Snail1 and Mef2c (Table 4.1). Samples were amplified for 35 cycles using Eppendorf Realplex (Eppendorf, Hamburg, Germany). The mRNA levels in relation to 28S ribosomal RNA were determined using a comparative C\textsubscript{T} method.

4.3.5 Ex vivo Endocardial Cushion Explant Culture

EndMT was assessed using endocardial cushion explants ex vivo [20,21]. Collagen (1 mg/ml, type I collagen from rat tail, BD Bioscience, Mississauga, ON, Canada) was prepared in M199 culture media (M5017, Sigma-Aldrich, Toronto, ON, Canada). Casted collagen was hydrated by OPTI-MEM media plus 1% of fetal bovine serum (FBS) and insulin-transferrin-selenium (ITS) for 30 minutes at 37 °C. Hearts were isolated from E10.5 Nox2\textsuperscript{-/-} and WT embryos. Atria and ventricular chambers were dissected out and the AV canal was cut open longitudinally and planted on the hydrated collagen gel with the endocardium facing the gel. Cushion explants were incubated at 37 °C overnight. The following day, M199 media with 10% of FBS were added to the cushion explants. To inhibit ROS production, heart explant cultures were treated with 5 mM NAC. The number of spindle shaped cells outgrowth from the explanted ventricles was quantified 4 days post culturing. Images were captured using phase contrast microscope (Observer D1, Zeiss, Germany).
4.3.6 Statistical Analysis

Data are presented as means ± SEM. Statistical analysis performed using Student t-test or two-way analysis of variance (ANOVA) followed by Bonferroni corrections (GraphPad Prism, version 5.0). Survival rate and incidence of congenital malformations was analyzed by Chi-square test. A $P$ value of less than 0.05 was considered statistically significant.
### Table 4.1. Primer sequences for real-time PCR analysis.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession No.</th>
<th>Product Size</th>
<th>Primer Sequence (5′ → 3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bmp2</td>
<td>NM_007553.3</td>
<td>151</td>
<td>F: CAAACACAAACAGCGGAAGC</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>R: CAGCAAGGGCAAAGGACAC</td>
</tr>
<tr>
<td>Bmp4</td>
<td>NM_007554.2</td>
<td>250</td>
<td>F: GTTATGAAGCCCCCAGCAGA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>R: CCCAATCTCCACTCCCTTGA</td>
</tr>
<tr>
<td>Gata4</td>
<td>NM_008092.3</td>
<td>134</td>
<td>F: GCCTGCGATGTCTGAGTGAC</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>R: CACTATGGGCACAGCAGCCT</td>
</tr>
<tr>
<td>Gata5</td>
<td>NM_008093.2</td>
<td>167</td>
<td>F: ACCCCACAACCTACCCAGCA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>R: GCCCTCACGGGAACTCCT</td>
</tr>
<tr>
<td>Mef2c</td>
<td>NM_001170537.1</td>
<td>405</td>
<td>F: CACCGAGTACAACGAGCCGCA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>R: CTGGTGCTGCAACGGATGTC</td>
</tr>
<tr>
<td>Nkx2.5</td>
<td>NM_008700.2</td>
<td>162</td>
<td>F: GACAGCGGCAGGACCAGACT</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>R: CTTTTAGCATAGGCCATG</td>
</tr>
<tr>
<td>Notch1</td>
<td>NM_008714.3</td>
<td>142</td>
<td>F: CAGCTTGCAACACCAGACAGA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>R: TAACGGAGTACGGCCCATGT</td>
</tr>
<tr>
<td>Snail1</td>
<td>NM_011427.2</td>
<td>114</td>
<td>F: CACACGCTGCCTTGTGTTC</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>R: GGTCAGAAAGCGACCGTT</td>
</tr>
<tr>
<td>Tbx5</td>
<td>NM_011537.3</td>
<td>103</td>
<td>F: AGGAGCAGGTGAGGCACAA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>R: GGGCCAGAGACACCATC</td>
</tr>
<tr>
<td>Tgf-β1</td>
<td>NM_011577.1</td>
<td>139</td>
<td>F: GCCCGAAGCGGACTACTATG</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>R: CACTGCTCCCGAATGTCT</td>
</tr>
<tr>
<td>Tgf-β2</td>
<td>NM_009367.3</td>
<td>230</td>
<td>F: CTGTGAGGTGAGGCTTCAC</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>R: GCAGGAGATGTGGGGGTCTTC</td>
</tr>
<tr>
<td>28S</td>
<td>NR_003279.1</td>
<td>178</td>
<td>F: GGGCCACTTTTGGTAAGCAG</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>R: TTGATTCCGCAGGGTGAGTTG</td>
</tr>
</tbody>
</table>

F: Forward, R: Reverse. Primers of Bmp4, Gata4, Gata5, Mef2c, Tbx5, Tgf-β1 and 28S were also used in Chapter 2.
4.4 RESULTS

4.4.1 Reduced Viability, Litter Size and Body Weight in Nox2−/−

Neonates

Mortality at birth in Nox2−/− mice was about 4 fold higher than WT neonates (16% vs. 4.6%) with diminishing survival rate during the first three weeks of life (P<0.001, Figure 4.1A). Litter size in Nox2−/− mice was smaller (P<0.05, Figure 4.1B), and their body weight at birth was significantly lower compared to WT controls (P<0.05, Figure 4.1C). A significantly lower in body size or growth retardation was observed in 24% of Nox2−/− embryos collected at E10.5-12.5 (Table 4.2, Figure 4.1D). Some of the embryos may undergo in utero absorption, as suggested by lower litter size in Nox2−/− mice.

4.4.2 Septal and Valve Defects in Nox2−/− Mice

Histological analysis of Nox2−/− hearts at P0 showed that 34% of Nox2−/− mice were born with various types of CHDs, which included 18% atrial septal defects (ASD) and 18% ventricular septal defects (VSD, Table 4.3, Figure 4.2A). Additionally, severe cases of septal malformation in the form of atrioventricular canal defects (AVCD) were observed in 3.3% of Nox2−/− neonates. Furthermore, 6.6% of Nox2−/− neonates had bicuspid aortic valves (BAV, Table 4.3, Figure 4.2 A). It should be noted that all cases of BAV were associated with septal abnormalities. Most Nox2−/− mice had a single ASD or VSD. However, 2 out of 61 Nox2−/− mice (3.3%) had both ASD and VSD. In addition, Nox2−/− hearts demonstrated valvular abnormalities. Specifically, the mitral and tricuspid
Figure 4.1. Survival and growth in Nox2<sup>−/−</sup> mice.

A) Mortality rate in Nox2<sup>−/−</sup> mice at birth and during the first three weeks of life compared to their age matched controls. N=129 in wild type (WT) group and N=112 in Nox2<sup>−/−</sup> group. B) Litter size at birth, N=10 or 13 litters per group. C) Body weight of neonates at birth, N=28 samples per group. D) Representative images of body size of WT and Nox2<sup>−/−</sup> mice at E10.5. Scale bar is 1 mm. *P<0.05, **P<0.001 vs. WT by Chi-square in A and unpaired Student t test in B and C.
Table 4.2. Number and percentage of embryos with growth retardation at E10.5-12.5.

<table>
<thead>
<tr>
<th></th>
<th>Number of litters</th>
<th>Growth Retardation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n/N)</td>
<td>(%)</td>
</tr>
<tr>
<td>WT</td>
<td>4</td>
<td>0/29</td>
</tr>
<tr>
<td>Nox2&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>4</td>
<td>6/25*</td>
</tr>
</tbody>
</table>

*P<0.01 vs. WT by Chi-square test.

Table 4.3. Incidence of congenital heart defects in Nox2<sup>-/-</sup> mice at P0.

<table>
<thead>
<tr>
<th></th>
<th>Wild-type (N=35 from 5 litters)</th>
<th>Nox2&lt;sup&gt;-/-&lt;/sup&gt; (N=61 from 12 litters)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>%</td>
</tr>
<tr>
<td>Normal</td>
<td>35</td>
<td>100</td>
</tr>
<tr>
<td>Abnormal</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>ASD</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>VSD</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>AVCD</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>BAV</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

ASD, atrial septal defect; VSD, ventricular septal defect; AVCD, atrioventricular canal defect. *P<0.05, **P<0.01 vs. wild-type by Chi-square analysis.
Figure 4.2. Congenital heart defects in Nox2−/− mice at P0.

A) Representative histological images of normal and abnormal hearts. Nox2−/− mice demonstrated ASD, AVCD, membranous and muscular (arrows) types of VSD, and BAV. B) Representative histological images of tricuspid and mitral valves. C) Representative histological images of aortic and pulmonary valves. D) Quantification of mitral and tricuspid valve length, E) quantification of mitral and tricuspid valve thickness. F) Quantification of aortic valve cell density. *P<0.05 vs WT, N= 9-12 per group, Scale bar in A is 200 µm and in B-C is 100 µm.
valves were shorter in length (Figure 4.2 B & D) and the distal tip of the mitral valve was bigger in size in Nox2\(^{-/-}\) mice \((P<0.05, \text{Figure } 4.2 \text{ B & E})\), but there was no significant difference in the thickness of distal region of tricuspid valves (Figure 4.2 B & E). Additionally, aortic valves of some Nox2\(^{-/-}\) mice had a bulging shape with lower cell density \((P<0.05, \text{Figure } 4.2 \text{ C & F})\) while no apparent abnormalities were found in the pulmonary valves.

### 4.4.3 NOX2 Expression Pattern and Endocardial Cushion Formation in Embryonic Heart

To examine the expression pattern of NOX2, immunohistochemical analysis was performed. NOX2 immunostaining was seen in the atrial and ventricular myocardium at E10.5 (Figure 4.3 A). Remarkably, NOX2 expression was more robust on the left ventricular myocardium compared to the right (Figure 4.3 C, E). Importantly, NOX2 was expressed in the myocardium covering endocardial cushions at the OFT and AV canal, suggesting their possible role in regulation of endocardial cushion development (Figure 4.3 D, F). However, the epicardium, endocardium and cells within the endocardial cushion do not express NOX2 in the WT hearts at E10.5 (Figure 4.3 C-E). As expected, in Nox2\(^{-/-}\) mice, NOX2 protein was not expressed (Figure 4.3 B, G), and the ROS levels were lower in the myocardium \((P<0.05, \text{Figure } 4.4)\). Furthermore, cellular density in endocardial cushion was significantly lower in Nox2\(^{-/-}\) compared to WT hearts at E10.5 (Figure 4.3 G).
Figure 4.3. Expression of NOX2 in E10.5 hearts.

A) Representative images of NOX2 immunostaining in embryonic heart of WT mice. B) Loss of NOX2 expression in Nox2<sup>−/−</sup> mice. C-E) Enlarged from A showing expression of NOX2 in right and left atrial and ventricular myocardium of WT mice. D &F) Expression of NOX2 in the myocardial cells covering the endocardial cushion at the AV canal and OFT of WT mice. G) Lack of NOX2 expression and lower cellular density were evident in OFT cushions of Nox2<sup>−/−</sup> hearts. Panels f and g are enlarged images of boxed area in F and G. Scale bar is 100 µm.
Figure 4.4. Analysis of superoxide levels in fetal hearts at E10.5 using dihydroethidium (DHE) as a probe.

A) Representative images of ROS levels in the left venticle myocardium. B) Quantification of DHE fluorescence intensity. Scale bar is 30 µm. Data are means ± SEM. N=3 hearts per group. *P<0.01 vs. WT by unpaired Student’s t test.
4.4.4 EndMT Is Impaired in Nox2⁻/⁻ Hearts

To investigate the role of NOX2 in endocardial cushion formation, we evaluated EndMT of endocardial cells in vivo and in vitro. To this end, expression levels of Snail1, an important marker of EMT [22], were analyzed. Our data show that Snail1 mRNA levels in the heart at E10.5 as well as the number of Snail1 positive cells in the AV cushion at E12.5 were lower in Nox2⁻/⁻ compared to WT embryos (P<0.05, Figure 4.5 A, C, D). To examine EndMT, the endocardial cushion of E10.5 fetal hearts was cultured on collagen gel and allowed for cell outgrowth for four days (Figure 4.5 B). The number of spindle shaped cells, which had undergone EndMT, were quantified. Nox2⁻/⁻ endocardial cushion had significantly lower in the number of spindle shaped cells compared to WT cushions (P<0.05, Figure 4.5 B, E). To lower ROS levels, wild type and Nox2⁻/⁻ endocardial cushions were treated with a ROS quenching agent, N-Acetylcysteine (NAC). Notably, treatment with NAC diminished EndMT in both WT and Nox2⁻/⁻ endocardial cushion (P<0.001, Figure 4.5 B, E).

4.4.5 Nox2 Deficiency Reduces Expression of Genes Crucial for EndMT

To further investigate the role Nox2 in regulating EndMT, we examined the expression of transcription factors and growth factors critical to EndMT and heart development in 10.5 hearts. Our data show that mRNA levels of Gata4, a transcription factor important in septal development, were diminished in Nox2⁻/⁻ mice. As well,
Figure 4.5. Analysis of endocardial EMT *in vivo* and *in vitro*.

A) Representative images of Snail1 expression in the endocardial cushion (EC) at E12.5. Lower panels are enlarged images of boxed areas in WT and Nox2−/−, respectively. B) EC *ex vivo* culture demonstrates EMT in the presence or absence of 5 mM NAC. Dashed line outlines cell migration border. C) Quantification of Snail1 positive cells in EC (N=4-5 hearts per group). D) Analysis of Snail1 mRNA expression levels at E10.5 fetal hearts (N=5 hearts per group). E) Quantification of the number of spindle shaped cells (N=4-5 hearts per group). Data are means ± SEM. *P<0.05 vs. untreated WT, **P<0.001 vs. untreated WT, †P<0.05 vs. untreated Nox2−/−. Scale bars are 100 and 20 µm in A, and 200 µm in B.
the expression levels of members of TGF-β superfamily, including \textit{Tgf-β2}, \textit{Bmp2} and \textit{Bmp4}, which are important regulators of endocardial cushion formation [23], were significantly lower in \textit{Nox2}^{-/} fetal hearts at E10.5 (\textit{P}<0.05, Figure 4.6 A-D). However, the expression levels of other cardiac transcription factors including \textit{Nkx2.5}, \textit{Tbx5}, \textit{Gata5} and \textit{Mef2c} were not altered in \textit{Nox2}^{-/} embryonic hearts (Figure 4.6 E-H). Additionally, the expression of \textit{Tgfβ1} and \textit{Notch1} [24-26] was not significantly different in \textit{Nox2}^{-/} hearts (Figure 4.6 I-J).

4.4.6 \textit{Nox2} Deficiency Increases Apoptosis and Reduces Proliferation Rate in Endocardial Cushions

ROS regulates cell proliferation and apoptosis in a variety of cell types. Using immunostaining of cleaved caspase-3, we analyzed cell apoptosis in the endocardial cushion at E10.5. \textit{Nox2} deficiency resulted in a 2 fold higher of apoptosis in the endocardial cushion (\textit{P}<0.05, Figure 4.7 A, C). We also assessed cell proliferation using \textit{Ki67} immunostaining. Our data show about 50% lower in cell proliferation rate in the endocardial cushion of \textit{Nox2}^{-/} compared to WT mice (\textit{P}<0.001, Figure 4.7 B, D). Furthermore, cellular density in the endocardial cushion of \textit{Nox2}^{-/} mice was significantly lower than the WT controls (\textit{P}<0.05, Figure 4.8 E).
Figure 4.6. Gene expression levels in fetal hearts at E10.5.

(A-D) The mRNA levels of Bmp4, Bmp2, Tgfβ2 and Gata4 in Nox2−/− fetal hearts were significantly lower than WT levels. (E-J) expression levels of Nkx2.5, Gata5, Mef2c, Tbx5, Notch1 and Tgfβ1 were not altered in Nox2−/− fetal hearts. Data are means ± SEM. N=7-9 hearts per group. At least two hearts were pooled for each sample. *P<0.01 vs. WT by unpaired Student’s t test.
Figure 4.7. Increased rate of apoptosis and reduced rate of proliferation in endocardial cushion (EC) of Nox2<sup>−/−</sup> hearts at E10.5 and E12.5 respectively.

A) Representative images of histological sections immunostained for cleaved caspase-3 in E10.5 hearts. Lower panels are enlargement of the boxed areas in upper panels. Arrows point to positive cells (Brown). B) Representative images of histological sections immunostained for Ki67 in E12.5 hearts. Lower panels are enlargement of the boxed areas of upper panels. C) Quantification of cleaved caspase-3 positive cells. D) Quantification of Ki67 positive cells. E) Quantification of EC cell density at E10.5. *P<0.05 vs. WT.
4.5 DISCUSSION

NOX2 is a major source of ROS production in cardiomyocytes [15]. It is well established that excessive ROS production is detrimental to normal embryonic development [27]. For example, increased ROS levels during pregestational diabetes result in CHDs in the offspring (Chapter 2). On the other hand, physiological levels of ROS function as a signalling molecule to promote cardiac differentiation and cardiomyocyte proliferation [14,28]. However, the role of Nox2-mediated ROS signalling in embryonic heart development remains unknown. The present study demonstrated that 34% of Nox2−/− neonates had CHDs including septal defects, AVCD, AV valve malformation, BAV, and impaired remodeling of aortic valves. Importantly, ROS deficiency resulted in lower EndMT in Nox2−/− endocardial cushions. Pharmacological inhibition of ROS production in vitro had even lower EndMT of endocardial cushions. Notably, Nox2 deficiency resulted in lower gene expression including Gata4, Tgfb2, Bmp2, Bmp4 and Snail1, which are required for EndMT and endocardial cushion development. Our study shows for the first time a critical role of NOX2-mediated ROS signalling in the regulation of EndMT and embryonic heart development (Figure 4.8).

EndMT is a critical process in the formation of endocardial cushions and valvoseptal development [29]. However, whether NADPH oxidase is essential in the regulation of EndMT during heart development remains unknown. The present study provides the following evidence in support of our hypothesis that NOX2-derived ROS signalling is required for EndMT during embryonic heart development. First of all, Nox2−/− embryos had a lower cellular density in the endocardial cushion at E10.5. Secondly, the
Figure 4.8. Schematic summary of NOX2 mediated ROS signaling on directing EndMT and valvoseptal development.

ROS release from NOX2 positively regulates transcript levels of factors that promote EndMT and formation of normal valvoseptal structures.
number of cells undergoing EndMT in endocardial cushion explants from \textit{Nox2}^{-/-} embryos was significantly lower. Importantly, treatment with the antioxidant N-Acetylcysteine had even lower EndMT in \textit{Nox2}^{-/-} cushion explants. Thirdly, the expression of EndMT regulators including \textit{Gata4}, \textit{Tgf-β2}, \textit{Bmp2} and \textit{Bmp4} was markedly lower in \textit{Nox2}^{-/-} hearts. Finally, \textit{Snail1} expression, a marker of cells undergoing EMT, was lower in the endocardial cushion of \textit{Nox2}^{-/-} embryos. Taken together, these data strongly support a critical role of NOX2-derived ROS signalling in the regulation of EndMT during embryonic heart development.

BMP2 and BMP4 are inductive signalling molecules that promote EndMT and are released from the myocardium in the AV canal and OFT regions [22]. Through activation of BMP receptors on endocardial cells, they increase the expression of \textit{Gata4}, \textit{Tgf-β2} and \textit{Snail1}, which are essential for EndMT [30]. In the present study, we demonstrated that NOX2 is expressed in the myocardium, which includes the AV canal and OFT myocardium, but not in the endocardium. This pattern of NOX2 expression allows NOX2-derived ROS to regulate BMP2/4 expression in the myocardium and subsequent TGF-β signalling in the endocardial cushion. Notably, ROS mediated TGFβ signalling in the regulation of EMT has been shown in many cell types including keratinocytes [31], alveolar cells [32] and mammary epithelial cells [33]. The decreased expression of \textit{Bmp2/4}, \textit{Tgf-β2}, and \textit{Snail1} in \textit{Nox2}^{-/-} hearts in the present study supports our hypothesis that Nox2-derived ROS promotes BMP/TGF-β signalling and EndMT in embryonic heart development.

Cell proliferation and apoptosis are key cellular events that regulate heart development during embryogenesis. It is generally believed that excessive levels of ROS
favor cell apoptosis [34]. Consistent with this notion, we recently showed that increased ROS production during pregestational diabetes reduces cell proliferation and increases apoptosis in the endocardial cushion. N-Acetylcysteine restored cell proliferation ration but not apoptosis (Chapter 2). Surprisingly, lowering ROS levels in control mice by N-Acetylcysteine treatment increases cell apoptosis (Chapter 2). In the present study, deficiency in Nox2 results in lower ROS in E10.5 hearts, higher cell apoptosis and lower cell proliferation in the endocardial cushion. Our findings suggest that physiological levels of ROS are critical to cell survival and proliferation during embryonic heart development. ROS signalling has been shown to induce developmental gene expression [34]. Thus, lower expression of Gata4, Bmp2/4 and Tgf-β2 observed in the present study may contribute to higher apoptosis and lower cellular density in the endocardial cushion of Nox2−/− hearts.

In summary, deficiency in Nox2 results in congenital defects of the septum and cardiac valves. We further demonstrated that a lack of NOX2-derived ROS production resulted in lower gene expression in the developing heart and disrupts EndMT with lower cell proliferation and higher apoptosis in the endocardial cushion. Our data show that NOX2 is essential in valvoseptal development (Figure 4.8). Notably, patients with chronic granulomatous disease (CGD) due to NOX gene mutations have ASDs [17]. Our study provides mechanistic insight into the development of congenital heart defects in CGD patients and supports a critical role of ROS signaling in embryonic heart development.
4.6 REFERENCES


5. CHAPTER 5

5.1 SUMMARY OF MAJOR FINDINGS

The overall objective of this thesis was to investigate the role of ROS on cardiac morphogenesis in mice. Specifically, I studied the effect of excess ROS levels on heart development in the offspring of mice with pregestational diabetes. We aimed to unravel the molecular mechanisms of cardiovascular defects induced by pregestational diabetes and provide new insight on potential therapeutic strategies. Additionally, I studied the role of basal ROS levels in cardiac morphogenesis in non-diabetic pregnancies in Nox2−/− mice, which are deficient in ROS production. Experimental approaches included histological analysis of heart structure, in vitro heart explant cultures and biochemical or molecular analysis of embryonic hearts.

In Chapter 2, I studied heart development in the offspring of females with pregestational diabetes and investigated therapeutic strategies to prevent abnormal heart development. To address this aim, an STZ-induced pregestational diabetes mouse model was employed. The offspring born to females with pregestational diabetes had lower litter size, lower body weight and higher mortality at birth. Importantly, a wide spectrum of CHDs including malformation of septal and conotruncal structures were observed in neonates. These malformations are comparable to CHDs in the offspring of patients with pregestational diabetes [1]. Increased incidence of CHDs was associated with oxidative stress in fetal hearts as evidenced by significantly higher ROS levels and diminished ratio of reduced to total glutathione levels. Molecular analysis demonstrated altered expression levels of factors critical to heart development including Gata4, Gata5 and Vegf-a. In
addition, apoptosis was increased in endocardial cushion and cell proliferation was markedly reduced in the embryonic hearts affected by maternal diabetes. Furthermore, I demonstrated for the first time that treatment of diabetic mice with an antioxidant, NAC, during gestation inhibits oxidative stress in fetal hearts, normalizes gene expression levels, improves cell proliferation, and decreases the incidence of CHDs in their offspring. The underlying mechanisms of NAC protection in maternal diabetes induced CHDs include reductions of ROS levels, elevations of glutathione levels, increases in cell proliferation, and restoration of altered gene expression profiles.

Formation of coronary arteries is pivotal to myocardial development. Abnormalities in formation of coronary arteries such as hypoplastic coronary artery disease increase the risk of sudden cardiac death in youth [2]. In Chapter 3, we demonstrated for the first time that excess ROS induced by pregestational diabetes led to coronary artery malformation in the offspring. Histological analysis of fetal and neonatal hearts exposed to maternal diabetes demonstrated lower diameter and volume of coronary arteries. Furthermore, pregestational diabetes resulted in abnormal epicardial formation as evidenced by reduced number of Wt1+ epicardial cells and lower cell proliferation rate. Additionally, the number of epicardial cells undergoing EMT was lower as evidenced by Wt1 lineage tracing, immunostaining and in vitro analysis of epicardial EMT in high glucose culture media. Notably, the expression of HIF1-α, which is a critical transcriptional regulator of Wt1, was lower in the fetal hearts. Furthermore, the expression of Wt1 downstream target genes such as ALDH1a2, bFGF, Snail and Slug was lower in the embryonic heart of diabetic dams. Remarkably, these abnormalities were all prevented by NAC treatment, leading to normal coronary artery development in
the offspring of diabetic dams. This study shows that maternal diabetes impairs coronary artery formation via elevating ROS levels, and provides a rationale for future clinical studies to investigate pregestational diabetes as a possible cause of hypoplastic coronary artery disease.

In Chapter 4, I aimed to study the role of basal ROS production in normal heart development. To achieve this goal, the NADPH oxidase Nox2−/− mouse model was utilized. Loss of Nox2 resulted in retardation of embryonic growth and lower survival rate in neonates. Importantly, deficiency of Nox2 resulted in abnormalities in AV septum and cardiac valves. In particular, Nox2 deficiency resulted in higher cell apoptosis and lower cell proliferation in the endocardial cushion. In addition, lack of Nox2 impaired EndMT as evidenced by endocardial cushion cultures ex vivo and lower cellular density in the endocardial cushion in vivo. Furthermore, treatment with NAC to reduce ROS levels resulted in lower EndMT in both wild type and Nox2−/− endocardial cushions. Importantly, Nox2 deficiency resulted in lower transcript levels of Tgf-β2, Bmp2, Bmp4, Snail1 and Gata4, which are essential in the induction and regulation of EndMT. Overall, this study demonstrates an important role of NOX2-mediated ROS release in EndMT and embryonic development of AV septum and cardiac valves.

In Summary, I have demonstrated a pivotal role of ROS signalling in embryonic heart development. My results show that normal physiological ROS production promotes cardiogenesis while excessive ROS levels seen in pathological conditions such as pregestational diabetes induce CHDs. Thus, maintaining a balance of ROS levels is of critical importance for embryonic heart development. Furthermore, these studies suggest
a therapeutic potential of NAC in the prevention of CHDs induced by pregestational diabetes. A summary of these findings is presented in Figure 5.1.

Figure 5.1. Schematic diagram on the role of ROS in regulating heart development in mice with pregestational diabetes and Nox2 deficiency.
5.2 STUDY LIMITATIONS

5.2.1 Justification of Diabetes Induced Animal Model

A variety of strategies have been employed to study mechanisms of embryopathy and CHDs induced by maternal diabetes in animal models. The techniques include glucose-induced diabetes [3,4], drug-induced diabetes [5-8] and genetic models that develop diabetes spontaneously [9]. Two important diabetogenic agents are alloxan and STZ [10,11], which are analogues of glucose and enter pancreatic beta cells via glucose transporter 2 (GLUT2). Intracellularly, they induce alkylation of DNA that ultimately leads to oxidative stress and cell death [12]. In comparison to alloxan, STZ maintains higher levels of drug stability, specificity and efficiency [13,14]. Diabetes induced by administration of diabetic agents is considered an acute form of type 1 diabetes, where the onset of diabetes occurs in about ten days following drug administration. To avoid the risk of STZ teratogenic effects on the fetus, STZ was administered to female mice prior to breeding. The biochemical characteristics of STZ induced diabetes may differ from clinical cases of patients that progress to diabetes over the years due to genetic changes or complications from immune deficiencies. An important difference between our pregestational diabetes mouse model and clinical cases is that diabetic mice did not receive insulin treatment before or during gestation. As a result, a much higher incidence of CHDs was observed in our model. Nevertheless, our mouse model demonstrates a wide spectrum of embryonic defects similar to patient cases. Thus, we believe this is an excellent mouse model for studying diabetic embryopathy [1].

The offspring of diabetic rodents induced by STZ demonstrate variable susceptibility to congenital malformation from no congenital malformation to minimal or
frequent cases [8,15,16]. Part of this variability is due to dose of STZ, animal age and the time of drug administration in relation to gestation. As an example, if embryos are affected by maternal diabetes at early gestation congenital defects could occur in central nervous system or cardiovascular structures and the embryo may demonstrate general retardation of growth [5]. However, embryos that are affected by maternal diabetes at late gestational age may suffer from macrosomia, childhood obesity and metabolic disorders [6]. In addition, variability exists among the offspring of the same litter, which were exposed to the same level of glucose. It has been suggested that genetic background or strain variability plays a major role in susceptibility of animals to diabetic embryopathy [17-19]. Based on our observations and previous reports, C57Bl/6 diabetic mice are resistance to neural tube defects [17], but are susceptible to CHDs (Chapter 2). The fact that morphological characteristics of CHDs are comparable to human cases suggests that the therapeutic strategies can be applied to clinical conditions.

5.2.2 Challenges in Understanding Causes of CHDs Induced by Diabetes

Reports of increased prevalence of congenital defects in infants of diabetic women in early 1950s [20,21] initiated several experimental studies to elucidate the cause of diabetic embryopathy. Animal models have been developed to characterize the birth defects and to investigate their molecular mechanisms [4,8,22,23]. These studies have shown that hyperglycemia induces biochemical disturbances including oxidative stress in embryos [24-27]. Advances in molecular analysis have expanded our knowledge of factors that are altered by maternal diabetes [28]. However, these studies did not fully clarify the molecular mechanisms of diabetic embryopathy.
A major challenge in our understanding of diabetic embryopathy is the variable abnormalities among individuals affected by similar maternal blood glucose levels. This phenomenon could arise from epigenetic effects of maternal diabetes on the developing embryo [29,30]. Epigenetic modifications are heritable without any changes in the genome coding sequence [31]. As an example, microarray analysis revealed altered expression of over a thousand genes that control transcription, organ morphogenesis or cell cycle regulation in the heart of diabetic embryos [32,33]. Many of these genes are target of epigenetic modifications such as histone methylation or acetylation, leading to inhibition or activation of gene expression [33]. Recent analysis showed increased intracellular glucose metabolism may elevate acetyl-CoA levels in the nucleus and lead to excess histone acetylation of DNA[34]. In a separate study, diabetic environment in utero modulates DNA methylation in the placenta of mice with pregestational diabetes [35]. Other than direct effects of diabetes on embryonic epigenetics, hyperglycemia may modify epigenetic patterns indirectly by altering the expression of chromatin modifying factors [36].

Another epigenetic factor, which may interfere with gene expression in diabetic embryopathy, are non-coding RNAs, such as microRNA. MicroRNA may hybridize with complementary mRNA sequences and interfere with their translation. Cell culture experiments demonstrated high glucose alters levels of microRNAs in vitro, thus interferes with translation of mRNAs [37]. In addition, retrospective analysis of genes that are altered by maternal diabetes revealed that majority of them are targets of at least one microRNA [33,38]. According to these studies hyperglycemia or high glucose conditions increase the risk of epigenetic alterations [39]. These results challenged
identifying a distinct molecular mechanism that defines a causal relationship between hyperglycemia-induced alterations of gene expression and embryonic defects. Nevertheless, the experimental analysis provided in this thesis demonstrates that antioxidant treatments normalize gene expression levels and reduced the incidence of CHDs. These results support our hypothesis that oxidative stress is the main cause of CHDs induced by maternal diabetes.

5.3 SUGGESTIONS FOR FUTURE RESEARCH

Insulin treatment to diabetic women reduces the risk of congenital defects in newborns [40,41]. Therefore, women with pregestational diabetes are strongly advised to plan their pregnancy and control their glucose levels by insulin injections or medications prior to conception and during gestation [42]. It should be noted that pregnancy alters glucose metabolism and insulin sensitivity, thus, achieving optimal glycemic control in diabetic women is challenging [43]. Because of this, the risk of birth defects in insulin treated diabetic women is still higher than non-diabetic population [44,45], indicating that insulin alone is not sufficient to protect against congenital defects in the offspring of diabetic women. In this thesis, we overcome this challenge by demonstrating the protective effect of NAC in reducing the risk of birth defects in the offspring of non-insulin treated pregestational diabetic mice. Future studies should also investigate the effects of insulin treatment on CHDs induced by pregestational diabetes in mice.

5.3.1 Therapeutic Potential of NAC on Prevention of CHD

Birth defects induced by pregestational diabetes are highly attributed to increased ROS levels and reduced antioxidant levels. Thus, an important therapeutic strategy of
diabetic embryopathy is replenishing antioxidant capacity of the embryo. Previous studies on diabetic embryopathy reported deficiencies of glutathione or antioxidant levels in embryos. Vitamins C, E and folic acid have the ability to scavenge free oxygen radicals [46,47] and their efficiency in diminishing embryonic malformation has been demonstrated in diabetic animal models [48-53]. NAC is a derivative of amino acid cysteine, which is an intracellular source of glutathione [54]. In pathological conditions where GSH levels are diminished, NAC treatment demonstrates higher efficiency and consistency in replenishing GSH levels in comparison to cysteine or glutathione. Oral treatment of glutathione does not increase plasma levels of cysteine or glutathione effectively due to enzymatic actions that hydrolyze glutathione in the digestive tract [55]. Likewise, cysteine oxidizes promptly in oxygenated extracellular environment, and only some cells have the ability to import the oxidized cysteine and reduce it [56]. In addition, systemic administration of cysteine shows disruptive effects on neurons of the central nervous system [57,58]. Remarkably, the acetyl group of NAC protects it against oxidation or hydrolysis and can efficiently elevate glutathione bioavailability [59]. NAC has been used in several pathological conditions to rescue GSH deficiency and inhibit oxidative stress [59]. Previous studies demonstrated *in vitro* or *in ovo* treatment of NAC reduces the rate of congenital defects in embryos induced by high glucose [60,61]. Additionally, treatment with GSH-ester decreases outflow tract defects induced by maternal diabetes [62]. We investigated whether oral treatment of NAC in diabetic females during gestation prevents the incidence of CHDs in their offspring. Remarkably, NAC treatment successfully elevated total and reduced GSH levels in fetal hearts of embryos of diabetic dams and prevented the development of CHDs (Chapter 2).
Additionally, NAC administration restores transcript levels in embryos affected by pregestational diabetes. Previous studies demonstrated that NAC can alter proteins activity and transcript levels of many genes [10]. Whether gene expression levels are directly affected by NAC or intracellular glutathione levels or as an indirect effect of reduced oxidative stress remains to be investigated. Further experimental analysis is also required to expand our knowledge on the pharmacokinetics of NAC prior to its application for clinical studies.

Although NAC is generally considered a safe antioxidant, cautions should be taken in prescribing the right dose of NAC to pregnant women. Our experimental analysis demonstrated a slight increase in the rate of CHDs (10%) in the control offspring by NAC treatment. Although this was not statistically significant due to a small sample size, it suggests that reduction of ROS levels below normal physiological levels may also be detrimental to normal heart development. Additionally, exposure to higher concentrations of NAC induces reductive stress, as evidenced by mitochondrial oxidation and elevated cytotoxicity in cultured cells [63]. In agreement with this notion, a high dose of NAC in rats has been shown to reduce fertility in females [64]. However, with the dose used in our studies, we did not observe any difference in pregnancy success rate between groups. Thus, dosage of NAC treatment should be carefully examined in further animal studies before initiating clinical trials.

NAC from the maternal circulation can cross the placenta and reach the developing embryos [65]. However, a possible impact of antioxidant activities of NAC on other maternal organs including kidneys, liver and pancreas should not be neglected. Our studies demonstrated that NAC has beneficial effects on metabolism as shown by
reduced food and water intake in the diabetic mice (Chapter 2). A small but non-significant reduction of blood glucose levels in diabetic females was also observed with NAC treatment. It is possible that NAC may have beneficial effects on insulin release or insulin sensitivity. Further studies are required to study these effects and associated molecular mechanisms of NAC.

As discussed before, maternal diabetes impairs embryonic development through multiple signalling pathways. Previous experimental studies demonstrated supplementation of folic acid, vitamin C or E reduces the risk of birth defects in diabetic pregnancies [48-53]. Here, we demonstrated that NAC is a potent molecule that protects embryos against oxidative stress induced by maternal diabetes. It is possible that a combination of folic acid, vitamins and NAC may have a synergistic benefit on the prevention of birth defects in the offspring of women with pregestational diabetes. To test this hypothesis, further pre-clinical and clinical studies are required to investigate the appropriate dose and their efficiency in preventing birth defects induced by maternal diabetes.

5.3.2 Role of NOX2 in Heart Development

NOX2 and NOX4 are expressed in both adult cardiomyocytes and cardiac stem cells, but are distinctly localized on the plasma membrane and mitochondrial cell membrane, respectively [66,67]. In Chapter 4, we demonstrated that NOX2 mediated ROS release regulates Tgf-β/Bmp signalling and promotes EndMT required for cardiac septum and valve development. Furthermore, we demonstrated during early heart development (E10.5) NOX2 expression pattern is more robust in the left ventricular myocardium compared to the right (Figure 4-3). While cellular and molecular
characteristics of cardiomyocytes of the right and left ventricle are distinct [68], understanding the regulators of this distinct gene expression profile become an essence in topics such as induction of embryonic stem cells differentiation to cardiomyocyte. Previous studies have demonstrated an important role of NOX4 in the regulation of cardiac stem cell differentiation [67]. Whether ROS release from NOX2 regulates cardiac differentiation in the same or a different mechanism is not known. Thus, an interesting extension to studies performed in Chapter 4 is to evaluate the role of ROS released from NOX2 or 4 in the regulation of factors essential for cardiomyocyte differentiation. This can be performed primarily by evaluation of temporal and spatial expression of NOX2 and NOX4 during cardiogenesis in the embryos. Followed by this, the effect of NOX2 expression or inhibition in embryonic stem cells in relation to gene expression levels has to be investigated. Distinct expression pattern of NOX proteins might imply different role of ROS released from each source. Further studies are required to examine the temporal and spatial expression of NOX family and their contributions to cardiomyocyte differentiation and proliferation.

5.4 CONCLUSION

This thesis provides evidence of a pivotal role of ROS signalling in the regulation of embryonic heart development. Here, I demonstrated that excess ROS levels induced by pregestational diabetes alter gene expression profiles required for cardiovascular development and thus lead to CHDs in the offspring. Additionally, I have shown that treatment with an antioxidant, NAC, restores altered gene expression and reduces the incidence of CHDs in the diabetic offspring. Furthermore, this thesis provided valuable insight into a critical role of basal ROS levels in promoting EndMT and heart
development. Overall, my doctoral research thesis has broadened our understanding of ROS levels on heart morphogenesis and our findings may have implications in the clinical prevention and treatment of CHDs during pregestational diabetes.

5.5 REFERENCE


practical use and potential risk to humans. J Diabetes Metab Disord 2013, 12(1):60.


APPENDIX A

Qingping Feng - eSirius Notification - Annual Protocol Renewal APPROVED by the AUS 2007-011-03::6

From: [Redacted]
To: [Redacted]
Date: 21/01/2014 9:11 AM
Subject: eSirius Notification - Annual Protocol Renewal APPROVED by the AUS 2007-011-03::6
CC: [Redacted]

2007-011-03::6:

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

Yearly Renewal Date: 12/01/2013

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

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

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

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

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