Research Article

NOX2 Is Critical to Endocardial to Mesenchymal Transition and Heart Development

Hoda Moazzen,1,2 Yan Wu,1,3 Anish Engineer,1 Xiangru Lu,1 Simran Aulakh,1 and Qingping Feng1,4

1Department of Physiology and Pharmacology, Schulich School of Medicine and Dentistry, Western University, London, Canada
2Institute of Molecular and Cellular Anatomy, Medical Faculty, Wendlingweg 2 RWTH Aachen University, Aachen, Germany
3Metabolic Syndrome Research Center, Second Xiangya Hospital, Central South University, Hunan, China
4Department of Medicine, Schulich School of Medicine and Dentistry, Western University, London, Canada

Correspondence should be addressed to Qingping Feng; qfeng@uwo.ca

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NADPH oxidases (NOX) are a major source of reactive oxygen species (ROS) production in the heart. ROS signaling regulates gene expression, cell proliferation, apoptosis, and migration. However, the role of NOX2 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 data show that 34% of Nox2-/− neonatal mice had various congenital heart defects (CHDs) including atrial septal defects (ASD), ventricular septal defects (VSD), atrioventricular canal defects (AVCD), and malformation of atrioventricular and aortic valves. Notably, Nox2-/− embryonic hearts show abnormal development of the endocardial cushion as evidenced by decreased cell proliferation and an increased rate of apoptosis. Additionally, Nox2 deficiency disrupted EndMT of atrioventricular cushion explants ex vivo. Furthermore, treatment with N-acetylcysteine (NAC) to reduce ROS levels in the wild-type endocardial cushion explants decreased the number of cells undergoing EndMT. Importantly, deficiency of Nox2 was associated with reduced expression of Gata4, Tgfβ2, Bmp2, Bmp4, and Snail1, which are critical to endocardial cushion and valvoseptal development. We conclude that NOX2 is critical to EndMT, endocardial cushion cell proliferation, and normal embryonic heart development.

1. Introduction

Congenital heart defects (CHDs) are birth defects in infants affecting about 1% of live births [1, 2]. The most common forms of CHDs are malformations of septal and valvular structures, accounting for more than 40% of the cases [3, 4]. An intricate network of signaling molecules and transcription factors in the epicardium, myocardium, and endocardium regulates cardiac morphogenesis [5]. While advances in genetic analysis have assisted in identifying genomic factors responsible for morphological abnormalities in patients with CHDs [6], only less than 20% of CHDs are attributed to chromosomal abnormalities or genetic mutations [7, 8]. Over 80% of CHDs have nongenetic or unknown causes that may involve various environmental factors such as maternal pregestational diabetes, obesity, and smoking [9], indicating our limited knowledge on factors that regulate cardiac morphogenesis.

Reactive oxygen species (ROS) are important signaling molecules that modulate the intracellular redox state and gene expression profiles to regulate cell proliferation, differentiation, apoptosis, and migration [10, 11]. An imbalance in ROS production may have adverse effects on fetal development [12, 13]. To this end, we and others have shown that higher ROS levels in the embryonic heart alter gene expression profile and result in a wide range of CHDs, suggesting that overproduction of ROS disturbs normal heart development [14–16].

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 atrioventricular (AV) canal regions [17]. With contributions from neural crest cells, endocardial cushions at OFT and AV canal form the aorticoventricular septum/semitunlar valves and AV valves/cardiac septum, respectively. EndMT is regulated by transcription factors and signaling molecules produced in the adjacent myocardium and endocardial cushions [18].

NADPH oxidases (NOX) are a family of O₂⁻ and H₂O₂-producing enzymes expressed in both phagocytic and non-phagocytic cells [19]. The enzyme complex is composed of NOX proteins (NOX1-5 and DUOX1-2), p22 phox, p40phox, p47phox, p67phox, 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 [20, 21]. Mutations of NOX genes result in chronic granulomatous disease, a rare condition occurring in 1:200,000-450,000 live births [22]. Notably, atrial septal defects are also seen in patients with chronic granulomatous disease [23], suggesting that mutations of NOX genes or a lack of NOX-derived ROS production may cause CHDs. However, the role of NOX enzymes in regulating cardiac morphogenesis and their underlying molecular mechanisms are not clear. In this study, we tested the hypothesis that deficiency of Nox2 disrupts EndMT and results in congenital septal and valvular defects. Our data show that Nox2⁻/⁻ mice exhibit cardiac septal defects and valvular abnormalities. Furthermore, deficiency of Nox2 impairs EndMT and AV endocardial cushion development. Our study reveals a critical role of NOX2-derived ROS signaling in EndMT and normal heart development.

2. Materials and Methods

2.1. Animals. Nox2⁻/⁻ (B6.129S-Cybb⁻/⁻ D2J, Stock No. 002365) and C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, Maine). Nox2⁻/⁻ mice were back-crossed to C57BL/6 background for more than ten generations; therefore, C57BL/6 mice were used as a control in all experiments. PCR analysis was performed to validate the Nox2 gene knockout model using the following primers: 5′ AAGAGAAACCTCTCTGTTGAA 3′ and 5′ GTTCTA ATTCCATCAAGCATTTACG 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 (8th edition, 2011). 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 Care Committee at Western University, Canada.

2.2. Heart Morphology and Immunohistochemistry. Heart morphology in postnatal day 0 (P0) mice was analyzed on serial heart sections under a microscope. Briefly, the mouse thorax was fixed in 4% paraformaldehyde overnight, dehydrated in ethanol, embedded in paraffin medium, and sectioned transversely to 5 μm serial sections. For assessment of AV valve thickness and length, serial sections of each heart were evaluated at P0. The longest and thickest region of each valve leaflet was quantified in at least 3 serial sections. E10.5 embryos were harvested to assess cell density in the AV cushion. For immunohistochemical staining, 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). Tissue sections were incubated with the following primary antibodies overnight: Ki67 (1:500, Abcam), activated caspase-3 (1:800, Cell Signaling), Snail1 antibody (1:300, Abcam) and NOX2 (1:500, BD Transduction Laboratories) followed by one of the following secondary antibodies (Vector Laboratories) 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) and visualized using 3,3′-diaminobenzidine tetrahydrochloride (Sigma-Aldrich). Heart sections were counterstained with modified Mayer’s hematoxylin (Thermo Scientific), and images were captured using a light microscope (Observer D1, Zeiss, Germany).

2.3. Analysis of ROS Levels. Frozen sections (10 μm) of E10.5 hearts were employed to assess superoxide levels using dihydroethidium (DHE) (Invitrogen Life Technologies, Burlington, Canada) as we previously described [15]. Briefly, heart sections were incubated with 2 μM DHE for 30 minutes in a humidified and light-protected chamber in room air at 37°C. DHE fluorescence signals were detected using a fluorescence microscope (Observer D1, Zeiss, Germany). Myocardial images (5 from each heart section) were captured using fixed exposure time for both groups. The intensity of fluorescence signals per myocardial area was quantified using AxioVision software.

2.4. Real-Time RT-PCR Analysis. Total RNA was extracted from E10.5 fetal hearts using a RNeasy Mini kit (Qiagen, Burlington, ON) as per manufacturer’s instructions. cDNA was synthesized using M-MLV reverse transcriptase. At least two hearts were pooled for each qPCR analysis. Real-time PCR was conducted using EvaGreen qPCR MasterMix (Applied Biological Materials, Vancouver, BC). Specific primers were designed for Ncx2.5, Gata4, Gata5, Tbx5, Bmp2, Bmp4, Tgfβ2, Snail1, and Mef2c (Table 1). Samples were amplified for 35 cycles using Eppendorf Realplex (Eppendorf, Hamburg). The mRNA levels in relation to 28S ribosomal RNA were determined using a comparative Ct method [15].

2.5. Ex Vivo Endocardial Cushion Explant Culture. Endocardial to mesenchymal transition (EndMT) was assessed ex vivo. AV cushions of similar size E10.5 embryos from Nox2⁻/⁻ and control dams were harvested and cultured on collagen gel. Collagen (1 mg/ml, type I collagen of rat’s tail, BD Biosciences) was prepared in M199 culture media (M5017, Sigma). 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.
The AV cushion regions together with the overlying myocardium were explanted, cut open, and seeded with the cushion side facing the collagen gel at 37°C overnight. The following day, the AV cushions adhered to the collagen gel and M199 media with 10% of FBS were added to the explants. To inhibit ROS production, heart explant cultures were treated with 5 mM N-acetylcysteine (NAC). The number of spindle-shaped cell outgrowth from the explanted cushions was quantified 3 days post culturing [24]. Phase contrast images were captured using an Observer D1 microscope (Zeiss, Germany).

2.6. Statistical Analysis. Data are presented as means ± SEM. Statistical analysis was performed using Student’s t-test or two-way analysis of variance (ANOVA) followed by a Bonferroni post hoc test. The survival rate and incidence of congenital malformations were analyzed by a Chi-squared test. A P value of less than 0.05 was considered statistically significant.

3. Results

3.1. Reduced Viability, Litter Size, and Body Weight in Nox2−/− Neonates. Litter size in Nox2−/− mice was smaller (P < 0.05, Figure 1(a)), and their body weight at birth was significantly lower compared to wild-type (WT) controls (P < 0.05, Figure 1(b)). A significant smaller body size or growth retardation was observed in 6 out of 25 (24%) Nox2−/− embryos collected at E10.5-12.5 while this was not seen in any of the 29 WT embryos (n = 4 litters per group, Figure 1(c)). It is possible that the embryos with drastic growth retardation die during gestation, explaining the 25% reduction in litter size at birth. Animal survival after birth was monitored for 21 days with Nox2−/− mice showing a significant lower survival compared to WT mice (72% vs. 92%, P < 0.001, Figure 1(d)).

3.2. Septal and Valve Defects in Nox2−/− Mice. Histological analysis of Nox2−/− hearts at P0 shows that 34% of Nox2−/− mice were born with various CHDs including atrial septal defects (ASD, 18%), ventricular septal defects (VSD, 18%), and severe cases of septal malformation in the form of atrioventricular canal defects (AVCD, 3.3%), which are septation defects (Table 2, Figure 2(a)). Furthermore, 6.6% of Nox2−/− neonates showed bicuspid aortic valves (BAV, Table 2, Figure 2(a)). Notably, 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. Nox2−/− hearts with septal defects also had malformations of atrioventricular valves (Figure 2(b)).

Table 1: Sequences of primers used for real-time PCR analysis.

| Gene | Accession No. | Product size (bp) | Primer sequence (5′ → 3′) |
|------|---------------|------------------|--------------------------|
| Bmp2 | NM_007553.3   | 151              | F: CAAACACAAAAGCCGGAAGC  |
|      |               |                  | R: CAGCAAGGGCAAAGGACAC   |
| Bmp4 | NM_007554.2   | 250              | F: GTTATGAGGCCCCACAGCAGA |
|      |               |                  | R: CCAAACTCCACTCCCTTGGA  |
| Gata4| NM_008092.3   | 134              | F: GCCCTGCGATGTCAGTGAATC |
|      |               |                  | R: CACTATGGGCAGAGGCTCT   |
| Gata5| NM_008093.2   | 167              | F: ACCCCACACCTACCCAGCAGA |
|      |               |                  | R: GCCCTACAGGGAACCTCCT   |
| Mef2c| NM_001170537.1| 405              | F: CACCGAGTACAGAGGCGGA   |
|      |               |                  | R: CAGCTGCTGCAAGCAGTTC   |
| Nkx2.5| NM_008700.2  | 162              | F: CAGAGCGGCAACCAAGCTCT  |
|      |               |                  | R: CGTTGATGCCATAGGCT     |
| Snail1| NM_011427.2  | 114              | F: AACACAGTGGCTGTGCTCT   |
|      |               |                  | R: GGTGCACAAAGACAGCAGA   |
| Tbx5 | NM_011537.3   | 103              | F: AGGAGGACAGTAGGGCACA   |
|      |               |                  | R: GGGGAGGAGACACATTTC    |
| Tgf-β2| NM_009367.3  | 230              | F: CGTGAGGAGGTGCTCAC     |
|      |               |                  | R: GCAGGAGATGTCGGTCTC    |
| Notch1| NM_008714.3  | 142              | F: CACCTGTGACAAACAGACAGA|
|      |               |                  | R: TAACGGAGTACGGCCATGT   |
| 28S  | NR_003279.1   | 178              | F: GGCGCACTTGTGGTTAGCCAG|
|      |               |                  | R: TTGATCGGCAGGTGAGT    |

F and R indicate forward and reverse primers, respectively.
3.4. 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, a marker of EMT [25], were analyzed. Our data show that Snail1 mRNA levels at E10.5 as well as the number of Snail1-positive cells in the AV endocardial cushion at E12.5 were lower in Nox2−/− compared to WT embryos (P < 0.05, Figures 5(a), 5(c), and 5(d)). To examine EndMT, the AV endocardial cushion of E10.5 fetal hearts was cultured on collagen gel and allowed for cell outgrowth for three days (Figure 5(b)). The number of spindle-shaped cells, which had undergone EndMT, was quantified. Nox2−/− endocardial cushions had a significantly lower number of spindle-shaped cells compared to WT cushions (P < 0.05, Figures 5(b) and 5(e)). To reduce ROS levels, WT and Nox2−/− AV cushion explants were treated with a ROS quenching agent, N-acetylcysteine (NAC). Notably, treatment with NAC further diminished EndMT of AV cushions in both WT and Nox2−/− samples (P < 0.001, Figures 5(b) and 5(e)).

3.5. Nox2 Deficiency Reduces Expression of Genes Crucial to Cushion Development. To further investigate the role of 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 to septal development, were diminished in Nox2−/− mice (P < 0.05, Figure 6(a)). Also, the expression levels of members of the TGF-β superfamily, including Tgf-β2, Bmp2, and Bmp4, which are important regulators of endocardial cushion formation [26], were significantly lower in Nox2−/− fetal hearts at E10.5 (P < 0.05, Figures 6(b)–6(d)). We also analyzed mRNA levels of other regulators of cardiac septum formation such as Nkx2.5, Gata5, Mef2c, Tbx5, TGFβ1, and Notch1; however, their expression levels were not significantly altered in Nox2−/− embryonic hearts (Figures 6(e)–6(j)).

3.6. Nox2 Deficiency Increases Apoptosis and Reduces Cell Proliferation in Endocardial Cushion. ROS regulates cell proliferation and apoptosis in a variety of cell types. Using immunostaining of cleaved caspase-3 protein, we analyzed cell apoptosis in the AV endocardial cushion at E10.5. Nox2 deficiency resulted in a 2-fold higher apoptosis in the AV endocardial cushion (P < 0.05, Figures 7(a) and 7(b)). We also assessed cell proliferation using Ki67 immunostaining (Figure 7(c)). Data was collected from 3–6 heart sections with

Table 2: Incidence of congenital heart defects in Nox2−/− mice at P0.

|                | Wild type (N = 35 of 5 litters) | Nox2−/− (N = 61 of 12 litters) |
|----------------|---------------------------------|---------------------------------|
|                | N  | %  | N  | %  |
| Normal         | 35 | 100| 40 | 65.6** |
| Abnormal       | 0  | 0  | 21 | 34.4** |
| ASD            | 0  | 0  | 11 | 18*   |
| VSD            | 0  | 0  | 11 | 18*   |
| AVCD           | 0  | 0  | 2  | 3.3   |
| BAV            | 0  | 0  | 4  | 6.6   |

ASD: atrial septal defect; VSD: ventricular septal defect; AVCD: atrioventricular canal defect. *P < 0.05, **P < 0.01 vs. wild-type by the Chi-squared test.
Figure 2: Congenital heart defects in Nox2<sup>-/-</sup> mice at P0. (a) Representative histological images of normal and abnormal hearts. Nox2<sup>-/-</sup> mice exhibited ASD, a complete AVCD (arrows point to interrupted septum primum and VSD), and membranous and muscular (arrows) types of VSD. (b) Representative histological images of tricuspid and mitral valves. (c) Toluidine blue staining of extracellular glycosaminoglycans (light purple) in AV valves. (d) Picrosirius red staining of collagen fibers. (e–g) Quantification of mitral and tricuspid valve length and AV valve thickness. (h) Quantification of the total glycosaminoglycan- (GAG-) positive area in AV valves. (i) Quantification of collagen deposition in mitral and tricuspid valves. *P < 0.05 and ***P < 0.001 vs. WT by Student's t-test. N = 9-12 per group. Scale bar in (a) is 200 μm and 100 μm in (b–d).
bulging AV endocardial cushion per heart, a total of 5 hearts per group. Our data show a 50% lower cell proliferation rate in the AV endocardial cushion of Nox2−/− hearts compared to WT embryos (P < 0.001, Figures 7(c) and 7(d)). Furthermore, cellular density in the AV endocardial cushion of Nox2−/− hearts was significantly lower than that of WT controls (P < 0.05, Figure 7(e)).

4. Discussion

EMT is a process by which epithelial cells undergo their phenotypic transformation to become mesenchymal cells [27, 28]. These multipotent mesenchymal cells are able to differentiate into a variety of cell types. Cells that undergo EMT lose their cell-cell junctions and epithelial cell polarity
Figure 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) Ex vivo EC culture demonstrates EMT in the presence or absence of N-acetylcysteine (NAC, 5 mM). Dashed line outlines cell migration border. (c) Quantification of Snail1-positive cells in EC at E12.5 (n = 4-5 hearts per group). (d) Analysis of Snail1 mRNA expression levels in E10.5 full hearts (n = 5 hearts per group). (e) Quantification of the number of spindle-shaped cells normalized to explant size (n = 3-5 hearts per group). Data are analyzed by unpaired Student’s t-test (c, d) and 2-way ANOVA followed by the Bonferroni test (e). *P < 0.05, **P < 0.001 vs. untreated WT; †P < 0.05 vs. untreated Nox2-/-.
followed by cytoskeletal reorganization and activation of the mesenchymal gene program. EMT is critical to many developmental, physiological, and pathological processes including organogenesis, wound healing, tissue fibrosis, and cancer metastasis. In heart embryogenesis, EndMT and epicardial EMT are essential for the development of cardiac valves/septum and coronary arteries, respectively [17, 29]. ROS derived from NADPH oxidases have been shown to promote EMT in numerous cell types. For example, ROS-mediated TGFβ signaling in the regulation of EMT has been shown in keratinocytes [30] and alveolar cells [31]. NOX2-derived ROS signaling has been shown to mediate the EMT process of human breast cancer cells [32]. NOX4 plays a role in TGFβ-driven EMT in lens epithelial cells to form myofibroblasts, resulting in cataract [33]. ROS signaling is critical to TGFβ1-induced renal tubular EMT in renal inflammation/fibrosis in angiotensin II-induced hypertension [34]. Additionally, ROS derived from NOX1 and NOX4 have been shown to drive cardiac differentiation and cardiomyocyte proliferation through regulating cardiac transcription factor expression [20, 35, 36]. However, the role of NOX2 in embryonic heart morphogenesis remains unknown. The present study demonstrated that one-third of Nox2-/- neonates had CHDs including septal defects, AVCD, and AV valve malformation. Importantly, Nox2 deficiency resulted in significantly lower EndMT and cell proliferation in endocardial cushions. Pharmacological inhibition of ROS production impaired EndMT in endocardial cushion explants. Furthermore, Nox2 deficiency reduced the expression of genes critical to AV cushion EndMT, cell proliferation/survival, and normal heart morphogenesis (Figure 8).

A network of signaling molecules and transcription factors in myocardial and endocardial cells regulates EndMT. TGFβ2 is expressed in the endocardium and myocardium at the AV and OFT regions during cushion formation [37].
BMP2 and BMP4 are released from the myocardium promoting EndMT in the AV canal [25]. Through activation of BMP receptors on endocardial cells, they increase the endocardial expression of \textit{Gata4}, \textit{Tgf-β2}, and \textit{Snail1}, which are essential for EndMT [38]. In the present study, we demonstrated that NOX2 is also expressed in the myocardium overlying the endocardial cushion. The proximity of NOX2 expression allows NOX2-derived ROS to regulate BMP2/4 expression in the myocardium and subsequent TGF-β signaling in the endocardial cushion. Interestingly, ROS also stimulate Snail1 transcription and EMT in mammary epithelial cells [39, 40]. Additionally, a reduction in ROS signaling from a knockdown of glucose-6-phosphate dehydrogenase, the enzyme that generates NADPH, impairs EMT and embryonic development in zebrafish [41]. The reduced AV cushion EndMT along with decreased expression of \textit{Bmp2/4}, \textit{Tgf-β2}, and \textit{Snail1} in \textit{Nox2}⁻/⁻ hearts in our study strongly suggest that NOX2-derived ROS promotes BMP/TGF-β signaling and AV cushion 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 [42]. Consistent with this notion, we recently showed that increased ROS production during pregestational diabetes reduces cell proliferation and increases apoptosis in the endocardial cushion [15]. N-Acetylcysteine treatment restored cell proliferation but not apoptosis. Surprisingly, lowering ROS levels in control mice by N-acetylcysteine treatment increases cell apoptosis [15]. In the present study, deficiency in \textit{Nox2} results in lower ROS in E10.5 hearts,

**Figure 7:** Apoptosis and cell proliferation in endocardial cushions. (a) Representative images of histological sections immunostained for cleaved caspase-3 protein at E10.5. Arrows point to positive cells (brown). (b) Quantification of cleaved caspase-3-positive cells at E10.5. (c) Representative images of Ki67 immunostaining in E12.5 hearts. Quantification of Ki67-positive cells (d) and cell density (e) in endocardial cushions at E12.5. Lower panels (scale bars =10 μm) are enlargement of the boxed areas of the upper panels (scale bars =100 μm) in (a, b). \( n = 5 - 7 \) hearts per group. * \( P < 0.05 \) vs. WT by unpaired Student’s \( t \)-test.
higher cell apoptosis, and lower cell proliferation in the endocardial cushion. These findings suggest that physiological levels of ROS are critical to cell survival and proliferation during embryonic heart development. ROS signaling has been shown to induce developmental gene expression [42]. Thus, lower expression of Gata4, Bmp2/4, and Tgf-β2 observed in the present study may lead to higher apoptosis and lower cellular density in the endocardial cushion of Nox2-/- hearts. The reduced cell proliferation and AV cushion EndMT in combination with higher apoptosis may contribute to CHDs in Nox2-/- mice.

A limitation of this study is the use of a whole body Nox2 knockout mouse. The possible impact of loss of NOX2 expression in tissues outside the cardiovascular system cannot be ruled out. For example, cardiac neural crest cells delaminated from the dorsal neural tube are critical to outflow tract development. It is likely that lack of NOX2 expression in the cardiac neural crest cells may impede their migration to the outflow tract leading to the bicuspid aortic valve, a hypothesis that needs to be further tested in future studies. Additionally, the Nox2-/- mice show a significant but relatively low penetrance of CHDs (34%). It is not clear if other NOX isoforms or ROS-producing enzymes are compensated for the global loss of Nox2 in this model. An extension to this study could be using tissue-specific and inducible animal models or generating animals with deficiency in both Nox2 and 4 genes.

In summary, deficiency in Nox2 results in congenital defects of the cardiac septum and valves. We further demonstrated that a lack of NOX2-derived ROS production decreases gene expression in the developing heart and disrupts AV cushion EndMT with lower cell proliferation and higher apoptosis in the endocardial cushion. Taken together, our study shows that endogenous ROS signaling from NOX2 is critical to normal heart development in mice. Importantly, our study provides a mechanistic insight into the pathogenesis of congenital heart defects in patients with chronic granulomatous disease [23].

Data Availability
The data that support the findings of this study are available from the corresponding author upon reasonable request.

Disclosure
A version of this manuscript was presented in a PhD thesis titled “Pregestational diabetes and congenital heart defects: role of reactive oxygen species,” Western University 2014.

Conflicts of Interest
The authors declare that they have no conflicts of interest.

Authors’ Contributions
H.M. and Q.F. conceptualized, designed, and wrote the manuscript. H.M., Y.W., and X.L. performed the experiments and analyzed data. S.A. performed the experiments. H.M. and A.E. helped in manuscript and figure preparation. All authors discussed the paper and contributed to data analysis.

Figure 8: Schematic summary of NOX2-mediated ROS signaling in directing EndMT and valvoseptal development. ROS production from NOX2 positively regulates the expression of factors that promote EndMT and formation of normal valvoseptal structures.
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