

Contents lists available at SciVerse ScienceDirect

Differentiation



journal homepage: www.elsevier.com/locate/diff

NOing the heart: Role of nitric oxide synthase-3 in heart development

Yin Liu, Qingping Feng*

Department of Physiology and Pharmacology, University of Western Ontario, London, Ontario, Canada N6A 5C1

ARTICLE INFO

Available online 11 May 2012

Keywords: Heart development Nitric oxide Endothelial nitric oxide synthase Congenital heart disease

ABSTRACT

Congenital heart disease is the most common birth defect in humans. Identifying factors that are critical to embryonic heart development could further our understanding of the disease and lead to new strategies of its prevention and treatment. Nitric oxide synthase-3 (NOS3) or endothelial nitric oxide synthase (eNOS) is known for many important biological functions including vasodilation, vascular homeostasis and angiogenesis. Over the past decade, studies from our lab and others have shown that NOS3 is required during heart development. More specifically, deficiency in NOS3 results in congenital septal defects, cardiac hypertrophy and postnatal heart failure. In addition, NOS3 is pivotal to the morphogenesis of major coronary arteries and myocardial capillary development. Interestingly, these effects of NOS3 are mediated through induction of transcription and growth factors that are crucial in the formation of coronary arteries. Finally, deficiency in NOS3 results in high incidences of bicuspid aortic valves, a disease in humans that often leads to complications with age including aortic valve stenosis or regurgitation, endocarditis, aortic aneurysm formation, and aortic dissection. In summary, these data suggest NOS3 plays a critical role in embryonic heart development and morphogenesis of coronary arteries and aortic valves.

© 2012 International Society of Differentiation. Published by Elsevier B.V. All rights reserved.

Contents

1.	Introduction	. 54
2.	Nitric oxide synthase	. 55
3.	NOS3 expression in the embryonic heart	. 55
4.	NOS3 and heart development	. 55
5.	NOS3 and its upstream activators	. 56
6.	NOS3 promotes cardiomyocyte proliferation	. 57
7.	NOS3 promotes postnatal heart maturation	. 57
8.	NOS3 and coronary artery development	. 57
9.	NOS3 and myocardial capillary development	. 58
10.	NOS3 and aortic valve development	. 58
11.	Future directions	. 59
12.	Conclusions	. 59
	Acknowledgments	. 60
	References	. 60

1. Introduction

Congenital heart disease refers to a defect in the structure of the heart and vessels present at birth. This defect, however, can be detected earlier by prenatal diagnosis such as fetal echocardiography. The disease affects 1–2% in the general population and is the leading

cause of death in infants during the first year of life in the industrialized countries (Casson et al., 1997; Cleves et al., 2003). It is estimated that 96,000 adults in Canada have congenital heart disease (Marelli et al., 2007). These patients have increased risk of arrhythmia, bacterial endocarditis and heart failure later in life (Borghi et al., 2007). Thus, congenital heart disease is a major cause of mortality and morbidity in both infants and adults. The underlying etiology of congenital heart disease may be either genetic or environmental, but is usually a combination of both. It is estimated that only 15% of all congenital heart disease can be traced to a known

^{*} Corresponding author. Tel.: +1 519 850 2989; fax +1 519 661 4051 *E-mail address*: qfeng@uwo.ca (Q. Feng).

^{0301-4681/\$-} see front matter © 2012 International Society of Differentiation. Published by Elsevier B.V. All rights reserved. Join the International Society for Differentiation (www.isdifferentiation.org) http://dx.doi.org/10.1016/j.diff.2012.04.004

cause (Borghi et al., 2007). Some genetic factors have been linked to cause congenital heart disease, while the majority of factors are still unknown. Thus understand the mechanisms that regulate the formation of the heart and the coronary artery system could further our understanding of the disease and lead to new strategies of its prevention and treatment. Nitric oxide is an important signaling molecule and produced during early embryonic development. In this review, the role of nitric oxide in embryonic heart development is examined and may help to shed some light on the morphogenesis of congenital heart disease.

2. Nitric oxide synthase

Nitric oxide (NO) is produced from the guanidino group of L-arginine in an NADPH-dependent reaction catalyzed by a family of NO synthase (NOS) enzymes in living organisms (Moncada et al., 1991; Kelly et al., 1996). Originally identified as a vasodilatory agent, NO is now recognized as an important signaling molecule involved in a wide range of physiological and pathophysiological processes including cell growth, apoptosis, antithrombosis, neurotransmission, and immunological regulation (Kelly et al., 1996; Razavi et al., 2005). There are 3 distinct isoforms of NOS: neuronal NOS (NOS1), inducible NOS (NOS2) and endothelial NOS (NOS3). Each NOS isoform is encoded by a distinct gene and is expressed in a variety of tissues and cell types. NO produced from NOS1 and NOS3 is involved in intracellular signaling, whereas the high output NO by NOS2 is associated with inflammatory processes (Kelly et al., 1996; Razavi et al., 2005). Shear stress induces NOS3 expression in the cardiovascular system during chick embryonic development (Groenendijk et al., 2005). In addition to shear stress, NOS3 can also be induced and activated by Krüppel-like factor 2 (Klf2), an endothelial transcription factor that is crucially involved in vasculogenesis (SenBanerjee et al., 2004; Dekker et al., 2005). In the adult heart, NO released from NOS3 has several major roles including coronary vasodilation and tonic inhibition of mitochondrial O2 consumption (Hare and Colucci, 1995; Kelly et al., 1996). NO may also play a role in the muscarinic-cholinergic inhibition of β -adrenergic-stimulated chronotropy (Balligand et al., 1993), inotropy (Hare and Colucci, 1995), and atrioventricular nodal conduction. The effects of NO are mediated by the cGMP-dependent signaling and protein modification through S-nitrosylation (Kelly et al., 1996; Sun et al., 2006).

3. NOS3 expression in the embryonic heart

NOS3 is expressed in the heart early during mammalian embryonic development (Bloch et al., 1999). Immunohistochemical analysis in mice using NOS3 specific antibodies revealed that the heart including cardiomyocytes start to express NOS3 at E9.5 and expression remains high up to E13.5. Starting from E14.5, levels of NOS3 expression decrease in both atria and ventricles. After E19.5 low NOS3 levels are still detectable and this level of NOS3 expression in the myocardium remains to adulthood (Bloch et al., 1999). Parallel with NOS expression, there are also changes in guanylate cyclase and cGMP production indicating the involvement of NO-cGMP signaling pathway in the heart development. The production of NO from NOS3 regulates the voltage-dependent L-type Ca²⁺ current in cardiomyocytes during early embryonic development and NO has been shown to promote cardiomyogenesis from mouse embryonic stem cells (Bloch et al., 1999; Ji et al., 1999; Kanno et al., 2004). It is important to note that although NOS1 (nNOS) and NOS2 (iNOS) are expressed during cardiogenesis, both NOS1 and NOS2 knockout animals have a normal cardiac phenotype.

4. NOS3 and heart development

Development of the four-chambered heart is a complex process involving migration, differentiation, proliferation and coordination of the cardiac progenitors. The cardiac progenitors are derived from three distinct fields, the anterior lateral plate mesoderm also known as the primary heart field, the second heart field which is located dorsal and anterior to the primary heart field, and the cardiac neural crest from the dorsal neural tube (Srivastava, 2006; Black, 2007; Gittenberger-de Groot et al., 2012). Additionally, the epicardium is also a source of cardiac progenitors which give rise to coronary arteries and possibly cardiomyocytes (Zhou et al., 2008: Gittenberger-de Groot et al., 2012). Cardiac morphogenesis starts from the anterior lateral plate mesoderm to form the cardiac crescent at embryonic day (E) 7.5 in the mouse embryo (Bruneau, 2002). By E8 the primitive heart (or heart tube) is formed. As the heart tube forms, cells from the second heart field migrate into the dorsal aspect to the heart tube in the pharyngeal mesoderm. Upon rightward looping of the heart tube, they cross the pharyngeal mesoderm and populate the outflow tract with contributions also from the cardiac neural crest. As development proceeds, the primitive heart undergoes chamber specification, septation and trabeculation. The atrial and ventricular septum starts to form at E10. Multiple primordia contribute to a central mesenchymal mass, including the mesenchyme on the leading edge of the primary atrial septum, the atrioventricular endocardial cushions, and the cap of mesenchyme on the spina vestibule (Webb et al., 1998; Briggs et al., 2012). Fusion of these components closes the ostium primum, completing atrial and atrioventricular septation. The mitral and tricuspid valves are derived from the endocardial cushion while the aortic and pulmonary valves are formed from endocardial cushions and with a contribution of neural crest cells (Armstrong and Bischoff, 2004). The formation of atrioventricular septum and valves is tightly regulated by coordinated cell proliferation, apoptosis and remodeling. By E14.5, a fully functional fourchambered heart is formed in mice (Bruneau, 2002).

Septal defects are the most common cardiac malformations in humans (Clark et al., 2006). However, the molecular mechanisms that govern the formation of atrial and ventricular septum remain poorly understood. In recent years, through the use of genetic knockout mice, several transcriptional factors including Gata4, Nkx2.5 and Tbx5 have been identified to be critical in the development of atrial and ventricular septum (Clark et al., 2006; Bruneau, 2008). In addition, mutations in these factors are associated with congenital septal defects in humans (Garg et al., 2003; Clark et al., 2006). Studies from our lab show that deficiency in NOS3 results in congenital septal defects (Fig. 1) and heart failure, and is accompanied by 85% postnatal mortality (Feng et al., 2002). All mortalities occur within the first 7 days after birth. Post-mortem examination shows $NOS3^{-/-}$ mice have a high incidence of congenital septal defects with 64% atrial septal defects and 11% ventricular septal defects. Congenital septal defects can be a result of improper fusion of the atrioventricular cushions during embryonic heart development. In addition, increases in apoptosis in atrioventricular cushions can also contribute to congenital septal defects (Bartram et al., 2001; Person et al., 2005). In order to investigate the role of NOS3 in septal development, myocardial apoptosis was analyzed in WT and $NOS3^{-/-}$ hearts at E12.5, a crucial time point in which the atrioventricular cushions start to fuse together. Our results show that there is an overall increase in the apoptotic activities in the $NOS3^{-/-}$ compared with WT hearts as measured by both caspase 3 activity and cytosolic DNA fragments. In order to determine the apoptotic activity in the atrioventricular cushion, TUNEL staining was used. Our data show that there is a significant increase in the apoptotic activity in the region of the atrioventricular cushions,



Fig. 1. Congenital septal defects in NOS3^{-/-} mice. (A) Gross morphology of neonatal hearts from WT (left), NOS3^{+/-} (middle), and NOS3^{-/-} (right) mice at P2. (B) through (D), Frontal sections of neonatal hearts. (B) Normal WT mouse heart, (C) through (E). Hearts from NOS3^{-/-} mice. (C) Atrial septal defect. (D) Ventricular septal defect. (E) Atrial and ventricular septal defects. Defects are indicated by arrows. RA, right atrium; RV, right ventricle; LA, left atrium; and LV, left ventricle. Reproduced from Feng et al. Circulation 2002 with permission from Wolters Kluwer Health.

which may contribute at least in part to the increased incidence of congenital septal defects seen in the postnatal day 1 NOS3^{-/-} mice (Feng et al., 2002). Low levels of NO produced by NOS3 inhibit apoptosis via S-nitrosylation of caspase-3, a critical protease responsible for the execution of apoptosis, and by promoting expressions of cytoprotective proteins including heme oxygenase-1, heat shock protein 70 (HSP70) and metallothionein (Razavi et al., 2005). In addition, NO has also been shown to inhibit mitochondrial cytochrome c release and decrease permeability transition pore opening via mitochondrial membrane depolarization and inhibition of Ca^{2+} accumulation (Brookes et al., 2000). These mechanisms may explain the increased apoptosis in the embryonic heart of NOS3^{-/-} mice.

Many factors may regulate the expression of NOS3 during embryonic heart development. Bio-informatics analysis of the mouse NOS3 locus showed three canonical Tbx5 binding sites, two of which are flanked by Gata binding site that binds to both Tbx5 and Gata4 with high affinity (Nadeau et al., 2010). Using genetically modified animals. Nadeau et al. (2010) showed that endocardial specific knock-down of Tbx5 results in atrial septal defects with a 100% penetrance, suggesting Tbx5, a potential upstream regulator of NOS3, is also crucial in the proper formation of the atrial septum. Furthermore, compound haploinsufficiency of Tbx5 and NOS3 exacerbates the cardiac phenotype caused by deletion of a single Tbx5 allele from endocardial cells, suggesting that NOS3 may be a genetic modifier of Tbx5 (Nadeau et al., 2010). Interestingly, decreases in enzyme activity and NO production in a 894 G > T polymorphism of the NOS3 gene are associated with increased risks of congenital heart disease (Veldman et al., 2002; Senthil et al., 2005; van Beynum et al., 2008). Environmental factors and maternal conditions including psychological stress, hypertension and diabetes have been linked to increased risks of congenital heart disease (Horne et al., 2004). It has been shown that these environmental and maternal conditions decrease NOS3 expression and/or activity (Andersen et al., 2009; Michel and Vanhoutte, 2010). The finding that NOS3, an enzyme regulated by environmental conditions, interacts with Tbx5, suggests the importance of gene-environment interactions in the setting of congenital heart disease and may help to explain the variable expressivity of the same mutation among affected family members and the complex inheritance patterns of congenital heart disease (Nadeau et al., 2010; van der Bom et al., 2010).

In addition to the congenital septal defect, cardiac hypertrophy is also seen in the P1 NOS3^{-/-} mice (Feng et al., 2002). From gross inspection and quantification, the NOS3 $^{-/-}$ hearts are significantly larger compared with WT hearts (Fig. 1A). In order to determine if the cardiac function is impaired, in vivo heart shortening was determined in the anesthetized P1 mice using ultrasound crystals that were placed on the surface of the heart. Our data show that percent shortening is significantly decreased in NOS3^{-/-} compared to WT hearts. In addition, the LV chamber size is significantly increased in NOS3^{-/-} hearts. Furthermore, severe pulmonary congestion with focal alveolar edema is also seen in NOS3^{-/-} mice at P1. Cardiac dysfunction and pulmonary congestion are typical clinical manifestations of heart failure. Our results suggest that the higher mortality in postnatal NOS3^{-/-} mice is likely due to the development of heart failure after birth (Feng et al., 2002). However, the exact cause of heart failure remains unclear. Congenital heart defects especially coronary artery malformation and decreased capillary density in the heart and lungs may contribute to the development of heart failure after birth in NOS3^{-/-} mice (Zhao et al., 2002; Han et al., 2004; Liu et al., 2010; Liu et al., 2011).

5. NOS3 and its upstream activators

In addition to transcription factors, NOS3 activity and expression are also regulated by a serine/threonine kinase Akt, also known as protein kinase B (PKB) (Burger et al., 2006; Dedkova et al., 2007; Zhang et al., 2007). Akt is an important mediator of phosphatidylinositol-3 kinase (PI3K) signaling that regulates a wide variety of cellular functions including survival, growth, proliferation, glucose uptake, metabolism, and angiogenesis (Shiojima and Walsh, 2006). The effects are achieved through the regulation of genes and proteins involved in these processes including NOS3 (Oudit et al., 2004; Chen et al., 2005). There are three Akt isoforms, Akt1, Akt2 and Akt3, which have similar structures and molecular sizes (57 kDa). All three Akt isoforms are expressed in the embryo starting from two-cell stage to virtually every organ during embryonic development. Inhibition of PI3K or Akt attenuates cardiomyocyte differentiation in embryonic stem cells, suggesting a critical role of PI3K/Akt signaling in early stage cardiomyocyte differentiation (Naito et al., 2003). Three Akt isoforms have similar but yet distinct physiological roles. Targeted disruption of the Akt1 gene in mice results in growth retardation with 20% reduction in body size as well as septal defects (Chen et al., 2001; Chang et al., 2010). Akt2 knockout mice display insulin resistance and growth retardation while Akt3 knockouts show reduced brain size (Cho et al., 2001; Easton et al., 2005). It is worth noting that the septal defects seen in the Akt $1^{-/-}$ resembles to that of the NOS $3^{-/-}$ mice. Akt $1^{-/-}$ mice show perimembranous and muscular ventricular septal defects as well as atrial septal defects which are similar to those of NOS3^{-/-} mice (Feng et al., 2002; Chang et al., 2010).

The effect of Akt is mediated in part by upregulation of Gata4 expression via phosphorylation of GSK-3 β (Morisco et al., 2001). GSK3β inhibits the DNA binding activity of Gata4. Upon phosphorylation by Akt, GSK3 β exits the nucleus, which curtails the inhibitory effect of GSK3^β on Gata4, leading to increases in Gata4 activation (Morisco et al., 2001). Gata4 is a transcriptional factor that belongs to an evolutionarily conserved family of zinc fingercontaining proteins, which has 6 members (Molkentin, 2000). Gata-1, -2 and -3 are expressed in hematopoietic stem cells while Gata-4, -5 and -6 are expressed in various mesoderm- and endoderm-derived tissues including the heart, liver, lung, gonad, and gut. Gata4 is a critical regulator of early cardiogenesis. Homozygous Gata4 knockout mice die at E9.5 and display defects in heart and foregut morphogenesis (Molkentin et al., 1997). Using tetraploid embryo complementation, Zhao et al. (2008) showed that Gata4 controls cardiomyocyte differentiation in mice. It appears that in the Gata $4^{-/-}$ mice, initiation of cardiomyocyte differentiation starts normally, but progenitors cannot differentiate into terminal cardiomyocytes as shown by the lack of smooth muscle actin, myosin heavy chain or sarcomeric actin staining (Zhao et al., 2008). In humans, heritable mutations of Gata4 result in congenital heart defects including, atrial septal defects, ventricular septal defects, atrioventricular septal defects, pulmonary valve thickening or insufficiency of cardiac valves (Garg et al., 2003). Interestingly, the promoter region of NOS3 contains Gata4 binding sites (Nadeau et al., 2010). Studies have shown that Gata4 binds to these sites and increases NOS3 promoter activity (Neumann et al., 2004), suggesting Gata4 also serves as an upstream activator of NOS3.

6. NOS3 promotes cardiomyocyte proliferation

NOS3 is important to cardiomyocyte proliferation during early postnatal cardiac development (Lepic et al., 2006; Hammoud et al., 2007). To assess proliferation, cardiomyocytes were isolated from neonatal mice born within 24 h and cultured for up to 96 h. Cell proliferation was determined by bromodeoxyuridine (BrdU) incorporation and cell counts. Our data showed that cultured NOS3^{-/-} cardiomyocytes displayed fewer cells and lower BrdU incorporation compared with WT cardiomyocytes (Lepic et al., 2006). In order to analyze if the decreased cardiomyocyte proliferation is related to the level of NO produced by NOS3, NOS3^{-/-} and WT cardiomyocytes were treated with an NO donor and a NOS inhibitor, respectively. Treatment with the NO donor, diethylenetriamine NONOate, increased BrdU incorporation and cell counts in NOS3^{-/-} cardiomyocytes. Inhibition of nitric oxide synthase activity using N^Gnitro-L-arginine methyl ester (L-NAME) decreased the level of BrdU incorporation and cell counts in WT cardiomyocytes. Furthermore, to investigate cardiomyocyte proliferation neonatal mice were treated with BrdU in vivo. Deficiency in NOS3 significantly decreased BrdU labeling indexes in neonatal hearts (Lepic et al., 2006). These data suggest that NO production from NOS3 is necessary for postnatal cardiomyocyte proliferation, providing a crucial role of NOS3 during postnatal heart development. However, despite decreases in cell proliferation, the overall size of the heart is enlarged in NOS3^{-/-} as compared to WT mice (Fig. 1A). This is probably due to the fact that cardiomyocyte proliferation is low and cardiac hypertrophy is the predominant response during early postnatal heart development (Li et al., 1996; Soonpaa et al., 1996).

7. NOS3 promotes postnatal heart maturation

The fetal and neonatal hearts develop through both hyperplasia and hypertrophy, which increases in cell number and cell mass, (Cluzeaut and Maurer-Schultze, 1986; Soonpaa et al., 1996). During early postnatal cardiac development, proliferation still occurs, albeit at a much lower level compared to fetal hearts. However, shortly after birth cardiomyocytes undergo binucleation, accompanied by a cessation of proliferation and a complete switch to hypertrophic growth (Li et al., 1996; Soonpaa et al., 1996). In rodents this transition from hyperplastic growth to hypertrophic growth occurs within approximately 1-2 weeks after birth (Li et al., 1996; Soonpaa et al., 1996). Embryonic heart development is characterized by the expression of cardiac specific proteins such as atrial natriuretic peptide (ANP) and proteins that form the contractile apparatus such as myosin heavy chain (MHC). After birth, as the heart gradually gains a mature phenotype, ANP expression is decreased and there is also a switch from beta to alpha isoform of MHC in cardiomyocytes (Houweling et al., 2005). In the mature myocardium, α -MHC is the predominant isoform (Lompre et al., 1984; Swynghedauw, 1986). Our study confirmed these postnatal changes that in the WT mouse hearts ANP expression is progressively decreased from postnatal day 1 to day 7 while the expression of α -MHC is increased (Lepic et al., 2006). On the other hand, in the NOS3 $^{-/-}$ hearts, the levels of ANP remain high and the levels of α -MHC are significantly decreased at postnatal day 7, suggesting that NOS3 deficiency disturbs the normal temporal changes of ANP and α -MHC during postnatal heart development. These data support the notion that NOS3 plays an important role in promoting postnatal heart maturation (Lepic et al., 2006). However, the underlying molecular mechanisms responsible for these changes are not fully understood. Studies have shown that NF-kB, a transcription factor that is involved in inflammation, cell survival and growth, can modulate the expression of ANP and α -MHC (Kobayashi et al., 2003; Pilz and Casteel, 2003). In addition, NO can increase or decrease the expression of NF-κB depending on its concentrations and the cell types (Bogdan, 2001; Pfeilschifter et al., 2001). It is possible that NO produced from NOS3 may act through the NF-kB pathway to modulate expressions of ANP and α -MHC in postnatal hearts.

8. NOS3 and coronary artery development

The development of the coronary vessels is an important event at the later stages of cardiogenesis. Coronary development starts at E9.5 in mice from proepicardial organ (PEO) located at the pericardial surface of the septum transversum close to the sinus venosus (Reese et al., 2002; Gittenberger-de Groot et al., 2012; van den Akker et al., 2012). Cells from PEO migrate and spread over the surface of the myocardium to become the epicardium. Concurrent with formation of the epicardium, some epithelial cells migrate further into the subepicardial space and undergo epithelial–mesenchymal transition (EMT) to become mesenchymal cells, which migrate throughout the developing myocardium and give rise to vascular smooth muscle cells, fibroblasts and possibly endothelial cells, and form coronary vasculature of the heart. The proximal ends of the coronary arteries connect to the ascending aorta through coronary orifices at the level of the left and right sinuses of the semilunar valves. Coronary veins connect to the right atrium via the coronary sinus. By E15.5, a complete coronary vascular system is established (Reese et al., 2002; Gittenberger-de Groot et al., 2012). Over the past decade, many factors have been shown to be crucial in the formation of the coronary arteries, but the role of NOS3 in this process is still not well understood.

Many factors have been shown to be involved in the coronary artery development. Gata4 is a master regulator of heart development (Laverriere et al., 1994; Crispino et al., 2001) and controls the expression of growth factors including vascular endothelial growth factor (VEGFa), basic fibroblast growth factor (bFGF) and erythropoietin (EPO) that are important for coronary vessel formation (Dame et al., 2004; Heineke et al., 2007). For example, inhibition of VEGF and bFGF decreases both capillary formation and arterial growth (Reese et al., 2002; van den Akker et al., 2008). Deficiency in EPO receptor resulted in thinning of the epicardium and disorganized coronary network and structure (Wu et al., 1999).

We recently demonstrated that neonatal $NOS3^{-/-}$ mice show severe abnormalities in coronary arteries (Liu et al., 2010). Furthermore, as early as E15.5, coronary artery diameters, density and volume were significantly decreased in the NOS3^{-/-} compared with WT embryos. In order to understand the molecular mechanism governing the correct formation of the coronary arteries, expression of transcription and growth factors critical to coronary development was analyzed in E12.5 hearts. Interestingly, the expression of Gata4. VEGFa. bFGF and EPO was down-regulated in the NOS3 $^{-/-}$ compared with WT embryonic hearts. Additionally, Wilm's tumor-1 (Wt1), a transcription factor critical for EMT and coronary artery formation, was also down-regulated and the number of epicardial derived cells (EPDC) was decreased in the NOS3 $^{-/-}$ hearts (Liu et al., 2010). These data suggest that NOS3 is critical to embryonic coronary artery development. However, the underlying molecular mechanisms are still not clear. It has been shown that NOS3 promotes proliferation in many cell types (Quinlan et al., 2000; Fukumura et al., 2006; Hammoud et al., 2007). For example, NOS3 promotes neonatal cardiomyocyte proliferation by inhibiting TIMP-3 expression (Hammoud et al., 2007). NOS3 also promotes migration of mesenchymal stem cells towards the heart (Li et al., 2009). Therefore it is possible that not only cell migration, but also cell proliferation of EPDCs is affected in the $NOS3^{-/-}$ mice. Thus, a combination of decreased transcription and growth factors and decreased ability of EPDC proliferation and migration into the myocardium may impair coronary artery development in the $NOS3^{-/-}$ mice.

9. NOS3 and myocardial capillary development

NOS3 is not only crucial in the formation of major coronary arteries, but also in the formation of capillaries and angiogenesis (Zhao et al., 2002). To investigate the role of NOS3 in capillary development, myocardial capillary densities were measured morphometrically in P1 mouse hearts. Our data showed that myocardial capillary densities were decreased in NOS3^{-/-} compared with WT mice (Fig. 2). Next, in vitro tube formation of cardiac endothelial cells was investigated on Matrigel. Lack of NOS3 impaired tube formation of endothelial cells. In order to analyze if the decreased tube formation is related to the level of NO produced by NOS3, NOS3^{-/-} and WT endothelial cells were treated with an NO donor and a NOS inhibitor, respectively.



Fig. 2. Myocardial capillary density in NOS3^{-/-} mice at P1. Heart sections were stained with Richardson's solution (A and B) and immunohistochemically stained for von Willebrand factor (C and D). A and C, abundant capillaries in the left ventricular (LV) myocardium of wild-type mice. (B and D) a few and scattered capillaries in the LV myocardium of NOS3^{-/-} mice. Reproduced from Zhao et al. (2002).

In vitro tube formation was inhibited by N^G-nitro-L-arginine methyl ester (L-NAME, a NOS inhibitor) in wild-type cells and restored by a NO donor, diethylenetriamine NONOate, in NOS3^{-/-} cells. In addition, angiogenesis was evaluated via implanting Matrigel in the myocardium of the adult heart. Following 3 days of implantation, significant angiogenesis is seen in the implanted Matrigel in WT mice. The angiogenic response was significantly decreased in the NOS3^{-/-} mice. These data show that deficiency in NOS3 impairs myocardial angiogenesis and capillary development, suggesting an important role of NOS3 in myocardial capillary development.

10. NOS3 and aortic valve development

The morphogenesis of the heart valves occurs concomitantly with changes in the cardiac morphology and in a complex process that includes initiation, cushion formation, elongation, valve remodeling and maturation (Butcher and Markwald, 2007; Hinton and Yutzey, 2011). Valve development is initiated during cardiac looping when the primary myocardium secretes a hyaluronan-rich matrix called cardiac jelly that projects into the lumen at the atrioventricular junction and the outflow tract at E9.0 in mice. The underlining myocardial cells produced factors including bone morphogenetic protein-2 (BMP2), BMP4 and transforming growth factor (TGF)- β , which activate the overlaying endocardium. At E10.5, the activated endocardial cells undergo EMT to become spindle shaped migratory cells (mesenchymal phenotype) and invade the cardiac jelly. Proliferation of the mesenchymal cells and matrix deposition extend the cushions into the cardiac lumen and form primordium of each distinct valve. This is then followed by elongation and remodeling/thinning of the valve primordium at E12.5, which leads to the gradual maturation of the valves that are rich in elastin, fibrillar collagen and proteoglycans. During valve remodeling, cell proliferation decreases, and subsequently there is little to no proliferation of valve interstitial cells in the adult. Formation of aortic and pulmonary valves is a result of septation of the outflow tract into aorta and pulmonary trunk, which gives rise to two semilunar valves, each consisting of three leaflets. Fusion of the atrioventricular canal endocardial cushions gives rise to mitral and tricuspid valves with 2 and 3 leaflets, respectively (Armstrong and Bischoff, 2004).

Normal aortic valves have 3 distinct cusps (or leaflets) while bicuspid aortic valves have only 2 cusps (Siu and Silversides, 2010). Lee et al. (2000) are the first to show that deficiency in NOS3 results in the formation of bicuspid aortic valves. The adult $NOS3^{-/-}$ mice had a high incidences (5/12, 42%) of bicuspid aortic valves (Lee et al., 2000). Subsequent studies confirmed bicuspid aortic valves with an incidence of about 30% in NOS3^{-/-} mice (Fernandez et al., 2009). In patients with bicuspid aortic valves, the most common subtypes are those with fusion of right and left coronary leaflets (R-L) and those with fusion of right and non-coronary leaflets (R-N) (Siu and Silversides, 2010). In $NOS3^{-/-}$ mice, the bicuspid aortic valve is the R-N subtype in which the left leaflet develops normally (Fernandez et al., 2009). Data from our lab also show that $NOS3^{-/-}$ mice have bicuspid aortic valves (Fig. 3). Furthermore, these mice have significant aortic regurgitation at P1 as demonstrated by pulsed-wave Doppler and color flow Doppler echocardiography (unpublished data). The data is consistent with aortic regurgitation seen in patients with bicuspid aortic valves (Chung et al., 2007).

Notch signaling is essential for early embryonic development of aortic valves and postnatal repression of calcium deposition in the aortic valves. Mutations in NOTCH1 gene cause autosomaldominant anomalies of aortic valves and severe valve calcification in humans (Garg et al., 2005). In mice, Notch1 transcripts are the most abundant in the developing aortic valves. Furthermore, Notch1 inhibits the activity of Runx2, a central transcriptional regulator of osteoblast cell fate. Thus, decreases in Notch1 signaling result in developmental defect in the aortic valve and postnatal calcification that causes progressive aortic valve disease (Garg et al., 2005). Additionally, Notch signaling is also required for the EMT process in endocardial cushions during early valve development (Niessen and Karsan, 2008). Interestingly, recent studies have shown that Notch signaling activates NOS3 and its downstream target, soluble guanylyl cyclase via PI3K/Akt pathway (Chang et al., 2011). Therefore, NOS3 is likely a potential downstream target of Notch signaling that governs aortic valve development and postnatal remodeling.



Fig. 3. Bicuspid aortic valves in NOS3^{-/-} mice at P1. Shown are representative images for tricuspid aortic valves in wild-type mice (right) and bicuspid aortic valves in NOS3^{-/-} mice (left). Lower panel is an enlargement of the boxed area of the upper panel. Scale bars: 350 μ m (upper panel), 190 μ m (lower panel).

11. Future directions

Over the past 12 years, several landmark studies have demonstrated a key role of NOS3 in embryonic heart development and its deficiency leads to congenital septal defects and bicuspid aortic valves (Lee et al., 2000; Feng et al., 2002; Fernandez et al., 2009). However, many questions remain to be addressed. For example, it is not known if a lack of NOS3 impairs mitral and tricuspid valve development. To this end, our preliminary data show that mitral and tricuspid valves are malformed in the NOS3^{-/-} mice, suggesting NOS3 also plays an important role in the embryonic development of atrioventricular valves (Liu et al., 2011). Additionally, lymph vessel system aids the maintenance of tissue fluid homeostasis and immune surveillance. Studies have shown that NOS3 mediates VEGF-induced lymphangiogenesis and, consequently, plays a critical role in lymphatic metastasis of cancer (Achen et al., 2005; Lahdenranta et al., 2009). However, the role of NOS3 in the embryonic development of lymph vessels remains to be determined. Furthermore, during development of the cardiac conduction system, Purkinje fibers receive paracrine cues from developing coronary vessels (Hyer et al., 1999; Mikawa and Hurtado, 2007). Inhibition of coronary artery development decreases Purkinje fiber differentiation suggesting that coronary arteries are necessary and sufficient for the induction of Purkinje fibers (Hyer et al., 1999). Thus, it is possible that coronary artery defects in $NOS3^{-/-}$ mice may impede the development of the cardiac conduction system (Liu et al., 2010). Whether a deficiency of NOS3 impairs development of cardiac conduction system remains to be investigated. Finally, NOS3 894G > T polymorphism, which results in reduced enzyme function (Veldman et al., 2002; Senthil et al., 2005), has been associated with human congenital heart disease including conotruncal defects, tricuspid atresia and atrioventricular septal defects (van Bevnum et al., 2008). Further studies are required to determine if this polymorphism is associated with congenital coronary anomalies and bicuspid aortic valves.

12. Conclusions

Cardiac NOS3 expression starts early in cardiogenesis at E9.5 and remain high until E13.5. This also coincides with the crucial period of rapid heart development and formation of coronary arteries and aortic valves. Deficiency in NOS3 results in cardiac



Fig. 4. Summary of cardiac phenotypes and possible underlying mechanisms in NOS3^{-/-} mice. AV, atrioventricular; EMT, epithelial-mesenchymal transition; BAV, bicuspid aortic valves.

hypertrophy, congenital septal defects, and postnatal heart failure. In addition, coronary arteries are malformed and myocardial capillary densities are decreased in NOS3^{-/-} mice. Furthermore, deficiency in NOS3 results in high incidence of bicuspid aortic valves. These data suggest that NOS3 plays a critical role in embryonic heart development and morphogenesis of coronary arteries and aortic valves (Fig. 4). Although how lack of NOS3 leads to these cardiac phenotypes is not completely understood, possible mechanisms may include increases in apoptosis, decreases in cell proliferation, changes in gene expression, and decreases in EPDC migration and EMT (Fig. 4). We anticipate that these new insights into the mechanisms of NOS3 during embryonic heart development may lead to therapeutic strategies in the prevention and treatment of congenital heart disease.

Acknowledgments

Studies in Feng lab are supported by grants from Heart and Stroke Foundation of Ontario (HSFO) and the Canadian Institutes of Health Research (CIHR). Dr. Feng is an HSFO Career investigator.

References

- Achen, M.G., McColl, B.K., Stacker, S.A., 2005. Focus on lymphangiogenesis in tumor metastasis 7, 121–127Cancer Cell 7, 121–127.
- Andersen, M.R., Simonsen, U., Uldbjerg, N., Aalkjaer, C., Stender, S., 2009. Smoking cessation early in pregnancy and birth weight, length, head circumference, and endothelial nitric oxide synthase activity in umbilical and chorionic vessels: an observational study of healthy singleton pregnancies. Circulation 119, 857–864.
- Armstrong, E.J., Bischoff, J., 2004. Heart valve development: endothelial cell signaling and differentiation. Circulation Research 95, 459–470.
- Balligand, J.L., Kelly, R.A., Marsden, P.A., Smith, T.W., Michel, T., 1993. Control of cardiac muscle cell function by an endogenous nitric oxide signaling system. Proceedings of the National Academy of Sciences USA 90, 347–351.
- Bartram, U., Molin, D.G., Wisse, L.J., Mohamad, A., Sanford, L.P., Doetschman, T., Speer, C.P., Poelmann, R.E., Gittenberger-de Groot, A.C., 2001. Double-outlet right ventricle and overriding tricuspid valve reflect disturbances of looping, myocardialization, endocardial cushion differentiation, and apoptosis in TGFbeta(2)-knockout mice. Circulation 103, 2745–2752.
- Black, B.L., 2007. Transcriptional pathways in second heart field development. Seminars in Cells and Developmental Biology 18, 67–76.
- Bloch, W., Fleischmann, B.K., Lorke, D.E., Andressen, C., Hops, B., Hescheler, J., Addicks, K., 1999. Nitric oxide synthase expression and role during cardiomyogenesis. Cardiovascular Research 43, 675–684.
- Bogdan, C., 2001. Nitric oxide and the regulation of gene expression. Trends in Cell Biology 11, 66–75.
- Borghi, A., Ciuffreda, M., Quattrociocchi, M., Preda, L., 2007. The grown-up congenital cardiac patient. Journal of Cardiovascular Medicine 8, 78–82.
- Briggs, L.E., Kakarla, J., Wessels, A., 2012. The pathogenesis of atrial and atrioventricular septal defects with special emphasis on the dorsal mesenchymal protrusion. Differentiation 84, 117–130.
- Brookes, P.S., Salinas, E.P., Darley-Usmar, K., Eiserich, J.P., Freeman, B.A., Darley-Usmar, V.M., Anderson, P.G., 2000. Concentration-dependent effects of nitric oxide on mitochondrial permeability transition and cytochrome c release. Journal of Biological Chemistry 275, 20474–20479.
- Bruneau, B.G., 2002. Transcriptional regulation of vertebrate cardiac morphogenesis. Circulation Research 90, 509–519.
- Bruneau, B.G., 2008. The developmental genetics of congenital heart disease. Nature 451, 943–948.
- Burger, D., Lei, M., Geoghegan-Morphet, N., Lu, X., Xenocostas, A., Feng, Q., 2006. Erythropoietin protects cardiomyocytes from apoptosis via up-regulation of endothelial nitric oxide synthase. Cardiovascular Research 72, 51–59.
- Butcher, J.T., Markwald, R.R., 2007. Valvulogenesis: the moving target. Philosophical Transaction of the Royal Society of London B: Biological Sciences 362, 1489–1503.
- Casson, I.F., Clarke, C.A., Howard, C.V., McKendrick, O., Pennycook, S., Pharoah, P.O., Platt, M.J., Stanisstreet, M., van Velszen, D., Walkinshaw, S., 1997. Outcomes of pregnancy in insulin dependent diabetic women: results of a five year population cohort study. British Medical Journal 315, 275–278.
- Chang, A.C., Fu, Y., Garside, V.C., Niessen, K., Chang, L., Fuller, M., Setiadi, A., Smrz, J., Kyle, A., Minchinton, A., Marra, M., Hoodless, P.A., Karsan, A., 2011. Notch initiates the endothelial-to-mesenchymal transition in the atrioventricular canal through autocrine activation of soluble guanylyl cyclase. Developmental Cell 21, 288–300.

- Chang, Z., Zhang, Q., Feng, Q., Xu, J., Teng, T., Luan, Q., Shan, C., Hu, Y., Hemmings, B.A., Gao, X., Yang, Z., 2010. Deletion of Akt1 causes heart defects and abnormal cardiomyocyte proliferation. Developmental Cell 347, 384–391.
- Chen, J., Somanath, P.R., Razorenova, O., Chen, W.S., Hay, N., Bornstein, P., Byzova, T.V., 2005. Akt1 regulates pathological angiogenesis, vascular maturation and permeability in vivo. Nature Medicine 11, 1188–1196.
- Chen, W.S., Xu, P.Z., Gottlob, K., Chen, M.L., Sokol, K., Shiyanova, T., Roninson, I., Weng, W., Suzuki, R., Tobe, K., Kadowaki, T., Hay, N., 2001. Growth retardation and increased apoptosis in mice with homozygous disruption of the Akt1 gene. Genes and Development 15, 2203–2208.
- Cho, H., Mu, J., Kim, J.K., Thorvaldsen, J.L., Chu, Q., Crenshaw 3rd, E.B., Kaestner, K.H., Bartolomei, M.S., Shulman, G.I., Birnbaum, M.J., 2001. Insulin resistance and a diabetes mellitus-like syndrome in mice lacking the protein kinase Akt2 (PKB beta). Science 292, 1728–1731.
- Chung, A.W., Au Yeung, K., Cortes, S.F., Sandor, G.G., Judge, D.P., Dietz, H.C., van Breemen, C., 2007. Endothelial dysfunction and compromised eNOS/Akt signaling in the thoracic aorta during the progression of Marfan syndrome. British Journal of Pharmacology 150, 1075–1083.
- Clark, K.L., Yutzey, K.E., Benson, D.W., 2006. Transcription factors and congenital heart defects. Annual Review of Physiolgy 68, 97–121.
- Cleves, M.A., Ghaffar, S., Zhao, W., Mosley, B.S., Hobbs, C.A., 2003. First-year survival of infants born with congenital heart defects in Arkansas (1993–1998): a survival analysis using registry data. Birth Defects Research A: Clinical and Molecular Teratology 67, 662–668.
- Cluzeaut, F., Maurer-Schultze, B., 1986. Proliferation of cardiomyocytes and interstitial cells in the cardiac muscle of the mouse during pre- and postnatal development. Cell Tissue Kinetics 19, 267–274.
- Crispino, J.D., Lodish, M.B., Thurberg, B.L., Litovsky, S.H., Collins, T., Molkentin, J.D., Orkin, S.H., 2001. Proper coronary vascular development and heart morphogenesis depend on interaction of GATA-4 with FOG cofactors. Genes Development 15, 839–844.
- Dame, C., Sola, M.C., Lim, K.C., Leach, K.M., Fandrey, J., Ma, Y., Knopfle, G., Engel, J.D., Bungert, J., 2004. Hepatic erythropoietin gene regulation by GATA-4. The Journal of Biological Chemistry 279, 2955–2961.
- Dedkova, E.N., Wang, Y.G., Ji, X., Blatter, L.A., Samarel, A.M., Lipsius, S.L., 2007. Signalling mechanisms in contraction-mediated stimulation of intracellular NO production in cat ventricular myocytes. Journal of Physiology 580, 327–345.
- Dekker, R.J., van Thienen, J.V., Rohlena, J., de Jager, S.C., Elderkamp, Y.W., Seppen, J., de Vries, C.J., Biessen, E.A., van Berkel, T.J., Pannekoek, H., Horrevoets, A.J., 2005. Endothelial KLF2 links local arterial shear stress levels to the expression of vascular tone-regulating genes. American Journal of Pathology 167, 609–618.
- Easton, R.M., Cho, H., Roovers, K., Shineman, D.W., Mizrahi, M., Forman, M.S., Lee, V.M., Szabolcs, M., de Jong, R., Oltersdorf, T., Ludwig, T., Efstratiadis, A., Birnbaum, M.J., 2005. Role for Akt3/protein kinase B gamma in attainment of normal brain size. Molecular Cell Biology 25, 1869–1878.
- Feng, Q., Song, W., Lu, X., Hamilton, J.A., Lei, M., Peng, T., Yee, S.P., 2002. Development of heart failure and congenital septal defects in mice lacking endothelial nitric oxide synthase. Circulation 106, 873–879.
- Fernandez, B., Duran, A.C., Fernandez-Gallego, T., Fernandez, M.C., Such, M., Arque, J.M., Sans-Coma, V., 2009. Bicuspid aortic valves with different spatial orientations of the leaflets are distinct etiological entities. Journal of the American College of Cardiology 54, 2312–2318.
- Fukumura, D., Kashiwagi, S., Jain, R.K., 2006. The role of nitric oxide in tumour progression. Nature Reviews Cancer 6, 521–534.
- Garg, V., Kathiriya, I.S., Barnes, R., Schluterman, M.K., King, I.N., Butler, C.A., Rothrock, C.R., Eapen, R.S., Hirayama-Yamada, K., Joo, K., Matsuoka, R., Cohen, J.C., Srivastava, D., 2003. GATA4 mutations cause human congenital heart defects and reveal an interaction with TBX5. Nature 424, 443–447.
- Garg, V., Muth, A.N., Ransom, J.F., Schluterman, M.K., Barnes, R., King, I.N., Grossfeld, P.D., Srivastava, D., 2005. Mutations in NOTCH1 cause aortic valve disease. Nature 437, 270–274.
- Gittenberger-de Groot, A.C., DeRuiter, M.C., Poelmann, R.E., 2012. The cardiovascular epicardium in development, disease and repair. Differentiation 84, 41–53.
- Groenendijk, B.C., Hierck, B.P., Vrolijk, J., Baiker, M., Pourquie, M.J., Gittenberger-de Groot, A.C., Poelmann, R.E., 2005. Changes in shear stress-related gene expression after experimentally altered venous return in the chicken embryo. Circulation Research 96, 1291–1298.
- Hammoud, L., Xiang, F., Lu, X., Brunner, F., Leco, K., Feng, Q., 2007. Endothelial nitric oxide synthase promotes neonatal cardiomyocyte proliferation by inhibiting tissue inhibitor of metalloproteinase-3 expression. Cardiovascular Research 75, 359–368.
- Han, R.N., Babaei, S., Robb, M., Lee, T., Ridsdale, R., Ackerley, C., Post, M., Stewart, D.J., 2004. Defective lung vascular development and fatal respiratory distress in endothelial NO synthase-deficient mice: a model of alveolar capillary dysplasia? Circulation Research 94, 1115–1123.
- Hare, J.M., Colucci, W.S., 1995. Role of nitric oxide in the regulation of myocardial function. Progress in Cardiovascular Diseases 38, 155–166.
- Heineke, J., Auger-Messier, M., Xu, J., Oka, T., Sargent, M.A., York, A., Klevitsky, R., Vaikunth, S., Duncan, S.A., Aronow, B.J., Robbins, J., Crombleholme, T.M., Molkentin, J.D., 2007. Cardiomyocyte GATA4 functions as a stress-responsive regulator of angiogenesis in the murine heart. The Journal of Clinical Investation 117, 3198–3210.
- Hinton, R.B., Yutzey, K.E., 2011. Heart valve structure and function in development and disease. Annual Review of Physiology 73, 29–46.

- Horne, B.D., Camp, N.J., Muhlestein, J.B., Cannon-Albright, L.A., 2004. Evidence for a heritable component in death resulting from aortic and mitral valve diseases. Circulation 110, 3143–3148.
- Houweling, A.C., van Borren, M.M., Moorman, A.F., Christoffels, V.M., 2005. Expression and regulation of the atrial natriuretic factor encoding gene Nppa during development and disease. Cardiovascular Research 67, 583–593.
- Hyer, J., Johansen, M., Prasad, A., Wessels, A., Kirby, M.L., Gourdie, R.G., Mikawa, T., 1999. Induction of Purkinje fiber differentiation by coronary arterialization. Proceedings of the National Academy of Sciences USA 96, 13214–13218.
- Ji, G.J., Fleischmann, B.K., Bloch, W., Feelisch, M., Andressen, C., Addicks, K., Hescheler, J., 1999. Regulation of the L-type Ca2 + channel during cardiomyogenesis: switch from NO to adenylyl cyclase-mediated inhibition. FASEB Journal 13, 313–324.
- Kanno, S., Kim, P.K., Sallam, K., Lei, J., Billiar, T.R., Shears 2nd, L.L., 2004. Nitric oxide facilitates cardiomyogenesis in mouse embryonic stem cells. Proceedings of the National Academy of Sciences USA 101, 12277–12281.
- Kelly, R.A., Balligand, J.L., Smith, T.W., 1996. Nitric oxide and cardiac function. Circulation Research 79, 363–380.
- Kobayashi, N., Mita, S., Yoshida, K., Honda, T., Kobayashi, T., Hara, K., Nakano, S., Tsubokou, Y., Matsuoka, H., 2003. Celiprolol activates eNOS through the PI3K-Akt pathway and inhibits VCAM-1 via NF-kappaB induced by oxidative stress. Hypertension 42, 1004–1013.
- Lahdenranta, J., Hagendoorn, J., Padera, T.P., Hoshida, T., Nelson, G., Kashiwagi, S., Jain, R.K., Fukumura, D., 2009. Endothelial nitric oxide synthase mediates lymphangiogenesis and lymphatic metastasis. Cancer Research 69, 2801–2808.
- Laverriere, A.C., MacNeill, C., Mueller, C., Poelmann, R.E., Burch, J.B., Evans, T., 1994. GATA-4/5/6, a subfamily of three transcription factors transcribed in developing heart and gut. The Journal of Biological Chemistry 269, 23177–23184.
- Lee, T.C., Zhao, Y.D., Courtman, D.W., Stewart, D.J., 2000. Abnormal aortic valve development in mice lacking endothelial nitric oxide synthase. Circulation 101, 2345–2348.
- Lepic, E., Burger, D., Lu, X., Song, W., Feng, Q., 2006. Lack of endothelial nitric oxide synthase decreases cardiomyocyte proliferation and delays cardiac maturation. American Journal of Physiology Cell Physiology 291, C1240–1246.
- Li, F., Wang, X., Capasso, J.M., Gerdes, A.M., 1996. Rapid transition of cardiac myocytes from hyperplasia to hypertrophy during postnatal development. Journal of Molecular and Cellular Cardiology 28, 1737–1746.
- Li, N., Lu, X., Zhao, X., Xiang, F.L., Xenocostas, A., Karmazyn, M., Feng, Q., 2009. Endothelial nitric oxide synthase promotes bone marrow stromal cell migration to the ischemic myocardium via upregulation of stromal cell-derived factor-1alpha. Stem Cells 27, 961–970.
- Liu, Y., Lu, M., Lu, X., Lacefield, J.C., Feng, Q. 2011. Deficiency in endothelial nitric oxide synthase results in congenital malformation of atrioventricular valves. FASEB Journal 25, 826.9.
- Liu, Y., Lu, X., Feng, Q., 2010. Deficiency in endothelial nitric oxide synthase impairs fetal coronary artery development in mic. Journal of Molecular and Cellular Cardiology 48, S94.
- Lompre, A.M., Nadal-Ginard, B., Mahdavi, V., 1984. Expression of the cardiac ventricular alpha- and beta-myosin heavy chain genes is developmentally and hormonally regulated. Journal of Biological Chemistry 259, 6437–6446.
- Marelli, A.J., Mackie, A.S., Ionescu-Ittu, R., Rahme, E., Pilote, L., 2007. Congenital heart disease in the general population: changing prevalence and age distribution. Circulation 115, 163–172.
- Michel, T., Vanhoutte, P.M., 2010. Cellular signaling and NO production. Pflugers Arch 459, 807–816.
- Mikawa, T., Hurtado, R., 2007. Development of the cardiac conduction system. Seminars in Cells and Developmental Biology 18, 90–100.
- Molkentin, J.D., 2000. The zinc finger-containing transcription factors GATA-4, -5, and -6. Ubiquitously expressed regulators of tissue-specific gene expression. The Journal of Biological Chemistry 275, 38949–38952.
- Molkentin, J.D., Lin, Q., Duncan, S.A., Olson, E.N., 1997. Requirement of the transcription factor GATA4 for heart tube formation and ventral morphogenesis. Genes Development 11, 1061–1072.
- Moncada, S., Palmer, R.M., Higgs, E.A., 1991. Nitric oxide: physiology, pathophysiology, and pharmacology. Pharmacological Reviews 43, 109–142.
- Morisco, C., Seta, K., Hardt, S.E., Lee, Y., Vatner, S.F., Sadoshima, J., 2001. Glycogen synthase kinase 3beta regulates GATA4 in cardiac myocytes. The Journal of Biological Chemistry 276, 28586–28597.
- Nadeau, M., Georges, R.O., Laforest, B., Yamak, A., Lefebvre, C., Beauregard, J., Paradis, P., Bruneau, B.G., Andelfinger, G., Nemer, M., 2010. An endocardial pathway involving Tbx5, Gata4, and Nos3 required for atrial septum formation. Proceedings of the National Academy of Sciences USA 107, 19356–19361.
- Naito, A.T., Tominaga, A., Oyamada, M., Oyamada, Y., Shiraishi, I., Monzen, K., Komuro, I., Takamatsu, T., 2003. Early stage-specific inhibitions of cardiomyocyte differentiation and expression of Csx/Nkx-2.5 and GATA-4 by phosphatidylinositol 3-kinase inhibitor LY294002. Experimental Cell Research 291, 56–69.
- Neumann, P., Gertzberg, N., Johnson, A., 2004. TNF-alpha induces a decrease in eNOS promoter activity. American Journal of Physiology: Lung Cellular and Molecular Physiology 286, L452–459.

- Niessen, K., Karsan, A., 2008. Notch signaling in cardiac development. Circulation Research 102, 1169–1181.
- Oudit, G.Y., Sun, H., Kerfant, B.G., Crackower, M.A., Penninger, J.M., Backx, P.H., 2004. The role of phosphoinositide-3 kinase and PTEN in cardiovascular physiology and disease. Journal of Molecular Cell Cardiology 37, 449–471.
- Person, A.D., Klewer, S.E., Runyan, R.B., 2005. Cell biology of cardiac cushion development. International Review of Cytology 243, 287–335.
- Pfeilschifter, J., Eberhardt, W., Beck, K.F., 2001. Regulation of gene expression by nitric oxide. Pflugers Archiv 442, 479–486.
- Pilz, R.B., Casteel, D.E., 2003. Regulation of gene expression by cyclic GMP. Circulation Research 93, 1034–1046.
- Quinlan, T.R., Li, D., Laubach, V.E., Shesely, E.G., Zhou, N., Johns, R.A., 2000. eNOSdeficient mice show reduced pulmonary vascular proliferation and remodeling to chronic hypoxia. American Journal of Physiology—Lung Cellular and Molecular Physiology 279, L641–650.
- Razavi, H.M., Hamilton, J.A., Feng, Q., 2005. Modulation of apoptosis by nitric oxide: implications in myocardial ischemia and heart failure. Pharmacology and Therapeutics 106, 147–162.
- Reese, D.E., Mikawa, T., Bader, D.M., 2002. Development of the coronary vessel system. Circulation Research 91, 761–768.
- SenBanerjee, S., Lin, Z., Atkins, G.B., Greif, D.M., Rao, R.M., Kumar, A., Feinberg, M.W., Chen, Z., Simon, D.I., Luscinskas, F.W., Michel, T.M., Gimbrone Jr., M.A., Garcia-Cardena, G., Jain, M.K., 2004. KLF2 Is a novel transcriptional regulator of endothelial proinflammatory activation. The Journal of Experimental Medicine 199, 1305–1315.
- Senthil, D., Raveendran, M., Shen, Y.H., Utama, B., Dudley, D., Wang, J., Wang, X.L., 2005. Genotype-dependent expression of endothelial nitric oxide synthase (eNOS) and its regulatory proteins in cultured endothelial cells. DNA Cell Biology 24, 218–224.
- Shiojima, I., Walsh, K., 2006. Regulation of cardiac growth and coronary angiogenesis by the Akt/PKB signaling pathway. Genes Development 20, 3347–3365.
- Siu, S.C., Silversides, C.K., 2010. Bicuspid aortic valve disease 55, 2789–2800. Journals of the American College of Cardiology 55, 2789–2800.
- Soonpaa, M.H., Kim, K.K., Pajak, L., Franklin, M., Field, L.J., 1996. Cardiomyocyte DNA synthesis and binucleation during murine development. American Journal of Physiology 271, H2183–2189.
- Srivastava, D., 2006. Making or breaking the heart: from lineage determination to morphogenesis. Cell 126, 1037–1048.
- Sun, J., Picht, E., Ginsburg, K.S., Bers, D.M., Steenbergen, C., Murphy, E., 2006. Hypercontractile female hearts exhibit increased S-nitrosylation of the L-type Ca²⁺ channel alpha1 subunit and reduced ischemia/reperfusion injury. Circulation Research 98, 403–411.
- Swynghedauw, B., 1986. Developmental and functional adaptation of contractile proteins in cardiac and skeletal muscles. Physiological Reviews 66, 710–771.
- van Beynum, I.M., Mooij, C., Kapusta, L., Heil, S., den Heijer, M., Blom, H.J., 2008. Common 894G > T single nucleotide polymorphism in the gene coding for endothelial nitric oxide synthase (eNOS) and risk of congenital heart defects. Clinical Chemistry and Laboratory Medicine 46, 1369–1375.
- van den Akker, N.M., Caolo, V., Wisse, L.J., Peters, P.P., Poelmann, R.E., Carmeliet, P., Molin, D.G., Gittenberger-de Groot, A.C., 2008. Developmental coronary maturation is disturbed by aberrant cardiac vascular endothelial growth factor expression and Notch signalling. Cardiovasc Research 78, 366–375.
- van den Akker, N.M.S., Caolo, V., Molin, D.G., 2012. Cellular decisions in cardiac outflow tract and coronary development; an act by VEGF and NOTCH. Differentiation 84, 62–78.
- van der Bom, T., Zomer, A.C., Zwinderman, A.H., Meijboom, F.J., Bouma, B.J., Mulder, B.J., 2010. The changing epidemiology of congenital heart disease. Nature Reviews Cardiology 8, 50–60.
- Veldman, B.A., Spiering, W., Doevendans, P.A., Vervoort, G., Kroon, A.A., de Leeuw, P.W., Smits, P., 2002. The Glu298Asp polymorphism of the NOS 3 gene as a determinant of the baseline production of nitric oxide. Journal of Hypertension 20, 2023–2027.
- Webb, S., Brown, N.A., Anderson, R.H., 1998. Formation of the atrioventricular septal structures in the normal mouse. Circulation Research 82, 645–656.
- Wu, H., Lee, S.H., Gao, J., Liu, X., Iruela-Arispe, M.L., 1999. Inactivation of erythropoietin leads to defects in cardiac morphogenesis. Development 126, 3597–3605.
- Zhang, Q.J., Li, Q.X., Zhang, H.F., Zhang, K.R., Guo, W.Y., Wang, H.C., Zhou, Z., Cheng, H.P., Ren, J., Gao, F., 2007. Swim training sensitizes myocardial response to insulin: role of Akt-dependent eNOS activation. Cardiovascular Research 75, 369–380.
- Zhao, R., Watt, A.J., Battle, M.A., Li, J., Bondow, B.J., Duncan, S.A., 2008. Loss of both GATA4 and GATA6 blocks cardiac myocyte differentiation and results in acardia in mice. Developmental Biology 317, 614–619.
- Zhao, X., Lu, X., Feng, Q., 2002. Deficiency in endothelial nitric oxide synthase impairs myocardial angiogenesis. American Journal of Physiology—Heart and Circulatory Physiology 283, H2371–2378.
- Zhou, B., Ma, Q., Rajagopal, S., Wu, S.M., Domian, I., Rivera-Feliciano, J., Jiang, D., von Gise, A., Ikeda, S., Chien, K.R., Pu, W.T., 2008. Epicardial progenitors contribute to the cardiomyocyte lineage in the developing heart. Nature 454, 109–113.