CHAPTER 5

The Cooperative Roles of Foxc1 and Foxc2 in Cardiovascular Development

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Abstract

Foxc1 and Foxc2 are closely related members of the Forkhead/Fox transcription factor family. The two *Foxc* genes have overlapping expression patterns in mesodermal and neural crest derivatives during development, as well as similar functions of gene regulation. Consistently, mouse mutants for each gene have similar abnormalities in multiple embryonic tissues, including the eye, kidney and cardiovascular system. Analysis of compound *Foxc1; Foxc2* mutant embryos reveals that the two *Foxc* genes have dose-dependent, cooperative roles in development. In particular, recent studies demonstrate that Foxc1 and Foxc2 are essential for arterial cell specification, lymphatic vessel formation, angiogenesis and cardiac outflow tract development. This chapter will summarize and discuss current knowledge about the function of Foxc1 and Foxc2 in cardiovascular development.

Introduction

The cardiovascular system is the first functional unit to form in the developing vertebrate embryo. The generation of the vasculature and heart requires a complicated series of morphogenetic interactions involving cells of several embryonic origins. Indeed, congenital cardiovascular defects represent the most common group of human birth defects, but the molecular mechanisms underlying the different anomalies still remain largely unknown. In particular, many studies must be completed to fully understand gene regulation associated with critical signaling pathways during cardiovascular development. This chapter will discuss recent findings concerning the cooperative and overlapping roles of Foxc1 and Foxc2 transcription factors in this complex developmental process.

FoxC1 and FoxC2 Proteins

Murine *Foxc1* (formerly *Mf1*) and *Foxc2* (formerly *Mfh1*) encode proteins with virtually identical DNA binding domains (97% identity; 99% similarity), while the N- and C- terminal flanking regions are somewhat diverse (56% and 30% homology, respectively). A duplication of the ancestral *FoxC* gene is likely to have taken place in deuterostomes,[1] as vertebrate species including frog, chicken, mouse and human, possess the two *FoxC* genes. Human *FOXC1* and *FOXC2* genes are located on chromosomes 6 (6p25) and 16 (16q22-q24), respectively, while mouse *Foxc1* and

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Forkhead Transcription Factors: Vital Elements in Biology and Medicine, edited by Kenneth Maiese.
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Foxc2 genes are located on chromosomes 13 and 8 in regions of conserved synteny between human and mouse, respectively (Mouse Genome Informatics; http://www.informatics.jax.org). In addition to in vitro DNA binding assays to determine the consensus DNA binding sequence for FOXC1,[2] NMR structural analysis suggests that the two FOXC proteins act as monomers and have the same binding specificity to target sequences.[3] Therefore, it is likely that the two proteins regulate the same downstream target(s) where they are co-expressed in the same cells.

Overlapping Expression of *Foxc1* and *Foxc2* during Development

While mouse *Foxc1* and *Foxc2* are not transcribed in the axial-mesoderm and its derivatives such as the notochord, they show largely overlapping domains of expression in many embryonic tissues that are derived from the nonaxial mesoderm, including the cardiovascular system.[4-13] For instance, expression of *Foxc1* and *Foxc2* is detected in endothelial and mesenchymal cells of the developing heart and blood vessels, second heart field (SHF) progenitors and their derivatives, as well as the proepicardium. Foxc1 and Foxc2 are expressed in arterial and venous endothelial cells of the developing blood vessels.[10] In addition, *Foxc1* and *Foxc2* are co-expressed in neural crest derivatives, including cells populated in the pharyngeal arches and the endocardial cushions of the cardiac outflow tract.[13,14] There are some regions in which expression domains of *Foxc1* and *Foxc2* do not overlap during development. Although transcripts of both *Foxc* genes are detected in neural crest-derived periocular mesenchyme surrounding the developing eye,[15,16] *Foxc1*, but not *Foxc2*, is expressed in mesoderm-derived periocular mesenchyme.[15] The onset of expression of the *Xenopus* homologue of *Foxc2* before gastrulation is earlier than that of the *Xenopus* homologue of *Foxc1*.[17]

Developmental Defects in *Foxc* Mutant Mouse Embryos

While this chapter focuses primarily on the functions of Foxc1 and Foxc2 in cardiovascular development, it should be noted that single mouse mutants for each gene similarly exhibit numerous developmental abnormalities in accordance with their broad expression patterns. A comprehensive summary of the phenotypes of single *Foxc* mutant mice, as well as compound *Foxc1; Foxc2* mutant mice, is given in Table 1.

Mice that are homozygous for either a spontaneous mutation in *Foxc1* (*congenital hydrocephalus*, $Foxc1^{ch}$) or an engineered null mutation ($Foxc1^{lacZ}$) die prenatally or perinatally with identical phenotypes.[7,18,19] These phenotypes include hemorrhagic hydrocephalus and multiple skeletal, ocular, genitourinary and cardiovascular defects, including the interruption or coarctation of the aortic arch, ventricular septal defects (VSD) and aortic and pulmonary valve dysplasia.[6-8,16,20-22] *Foxc1* mutant mice also lack the frontal bones of the skull vault, derivatives of cranial neural crest cells.[23] Endothelial-specific *Foxc1* mutant mice have recently been generated and survive into adulthood, but these conditional *Foxc1* mutant mice have defects in the postnatal microvasculature (Table 1).[24,25] In addition, a forward genetic screen using ethylnitrosurea (ENU) mutagenesis has recently identified a hypomorphic mouse allele for *Foxc1* (*hole-in-the-head*, $Foxc1^{hith}$) that survives into adulthood.[26] A missense mutation in the $Foxc1^{hith}$ allele results in a Phe-to-Leu substitution at amino acid 107 within the second helix of the DNA binding domain, leading to destabilization of the protein. Analysis of $Foxc1^{hith}$ has revealed that Foxc1 plays a role in meaningeal differentiation, thereby regulating cortical development.

Foxc2 null mutants also die pre or perinatally with skeletal, genitourinary and cardiovascular defects similar to those seen in *Foxc1* homozygous mutants.[5,6,8,12,13,27] It is of interest to note that Foxc2 has been implicated in lymphatic vessel development. Heterozygous *Foxc2* mutant mice have hyperplasia of lymphatic vessels,[28] while homozygous *Foxc2* mutant mice show defective lymphatic valves and abnormal pericyte recruitment of lymphatic vessels.[29] These abnormalities underlie congenital defects caused by *FOXC2* mutations in humans (see below). Additionally, endothelial cells isolated from heterozygous *Foxc2* mutant mice exhibit impaired formation of microvessels.[30] Together, these findings demonstrate that Foxc1 and Foxc2 are required for mammalian embryonic development, including the cardiovascular system.

Table 1. Developmental defects found in Foxc mouse mutants

Mutant	Phenotype	References
Foxc1$^{+/-}$ (*Foxc1*$^{ch/+}$)	Delayed calvarial formation; Anterior segment abnormalities in the eye	19,21,22
Foxc1$^{-/-}$ (*Foxc1*$^{ch/ch}$)	Hydrocephalus; Malformations in the cranial and axial skeleton; Duplex kidneys and double ureters; Impaired gonad development; Anterior segment abnormalities in the eye	6,7,16,18, 19,20,21,76
Foxc1$^{hith/hith}$	Hydrocephalus; Incomplete skull closure; Cortical dysplasia; Microphthalmia	26
Conditional *Foxc1* (Endothelial specific)	Reduced migration of endothelial cells; Reduced expression of *CXCR4* and *Hey2*	24,25
Foxc2$^{+/-}$	Hyperplastic lymphatic vessels; Extra eyelashes; Anterior segment abnormalities in the eye; Impaired functions of microvessels	22,28,30
Foxc2$^{-/-}$	Malformations in the cranial and axial skeleton; Hypoplastic kidneys; Abnormal aortic arch patterning; VSD; Increased pericyte investment and agenesis of valves of lymphatic vessels; Abnormal glomerular development	5,6,12,27, 29,76
Foxc1$^{+/-}$; *Foxc2*$^{+/-}$	Hypoplastic kidneys and a single hydroureter; Abnormal aortic arch patterning; VSD; Anterior segment abnormalities in the eye	6,13,22
Foxc1$^{-/-}$; *Foxc2*$^{+/-}$	Die at around E12.5; Etiology of lethality is not determined	8,10,11,37
Foxc1$^{+/-}$; *Foxc2*$^{-/-}$	Small somites; expansion of intermediate mesoderm; Impaired remodeling of blood vessels; Hypoplastic OFT; Apoptotic neural crest; Abnormal epicardium	8,10,11,37
Foxc1$^{-/-}$; *Foxc2*$^{-/-}$	No somites formed; Expansion of intermediate mesoderm; Impaired remodeling of blood vessels; Disrupted arterial cell specification; Absence of the OFT; Apoptotic neural crest	8,10,11,37

Mutations in *FOXC1* and *FOXC2* Genes Associated with Developmental Disorders in Humans

Consistent with the importance of *Foxc* genes in murine development, mutations of human *FOXC* genes have been found in individuals with congenital anomalies. Mutations of the human *FOXC1* are associated with the dominantly inherited Axenfeld-Rieger anomaly (ARA), characterized by anterior chamber dysgenesis in the eye and congenital glaucoma [Online Mendelian Inheritance in Man (OMIM) no. 601090].[31,32] *Foxc1*+/– mice exhibit ocular abnormalities similar to those seen in human ARA patients, but these *Foxc1* heterozygous mice do not show increased intraocular pressure, the most important risk factor for glaucoma.[22] Some humans heterozygous for mutations in *FOXC1* have congenital heart defects such as mitral valve dysplasia and atrial septal defects.[13,31,33]

Mutations in human *FOXC2* are responsible for the autosomal dominant syndrome, Lymphedema-distichiasis (LD), characterized by the obstruction of lymphatic drainage of the limbs and the growth of an extra set of eyelashes (OMIM no. 602402).[34] In addition to lymphatic valve failure, mutations of human *FOXC2* are also associated with venous valve failure,[35] while

~15% of these humans exhibit conotruncal cardiac defects.[34] Although *Foxc2+/–* mice have similar ocular abnormalities as those seen in *Foxc1+/–* mice,[22] LD patients with *FOXC2* mutations present mild anterior chamber abnormalities unassociated with glaucoma.[36] Mutations of human *FOXC1* or *FOXC2* are likely to lead to haploinsufficiency and no individuals that are homozygous for a mutation of either *FOXC1* or *FOXC2* have been found. Elucidation of overlapping functions of the two *FoxC* genes required genetic analysis of compound *Foxc1; Foxc2* mutant mice.

Cooperative Roles of Foxc1 and Foxc2 in Cardiovascular Development

To determine functional interactions between Foxc1 and Foxc2, the generation of compound *Foxc1; Foxc2* mutant mice was performed. It is remarkable that the majority of compound *Foxc1; Foxc2* heterozygous mice from crosses of *Foxc1+/–* and *Foxc2+/–* mice die pre or perinatally, whereas only a small percent of the compound heterozygotes survive into adulthood. Most compound heterozygotes show a similar spectrum of cardiovascular, genitourinary and eye abnormalities like those seen in each single homozygous null mutant.[6,13,22] These include interruption/coarctation of the aortic arch, VSD, dysplasia of the aortic and pulmonary valves and an abnormally thin myocardium. Thus, these findings demonstrate that the two *Foxc* genes functionally overlap and cooperate with each other in vivo and that they play dose-dependent roles in many aspects of embryonic development.

From crossing fertile compound *Foxc1; Foxc2* heterozygotes, compound homozygous and hetero/homozygous embryos have been obtained and analyzed.[8,10,11,37] While compound *Foxc1+/–; Foxc2–/–* and *Foxc1–/–; Foxc2+/–* mutants die at embryonic day 12.0-12.5 (E12.0-12.5), compound homozygous embryos die around E9.5 with a phenotype that is much more severe than that of a single homozygote, a compound heterozygote, or a compound hetero/homozygous mutant (Table 1). As described below, compound homozygous embryos show disrupted arterial specification.[10] In addition, compound *Foxc1+/–; Foxc2–/–* mutants have a reduction in the number of Prox1-positive lymphatic endothelial cells sprouting from the cardinal vein.[10] Compound *Foxc1; Foxc2* mutants also have a wide spectrum of early cardiac abnormalities in a dose-dependent manner.[11] These observations further reinforce the idea of gene-dosage effects of the two *Foxc* genes during cardiovascular development. Of interest, zebrafish has only one *foxC* gene, *foxC1*, although, due to genome duplication, there are two zebrafish homologues, *foxC1.1* and *foxC1.2*.[38] Given that *foxC2* appears to be absent from the zebrafish genome,[39,40] knockdown of *foxC1.1*, but not *FoxC1.2*, in zebrafish results in the lack of segmented somites,[41] a phenotype similar to that seen in compound *Foxc1; Foxc2* homozygous mouse mutants (Table 1).[8] It is therefore likely that the cooperative roles of the *FoxC* genes in development are conserved in vertebrates.

Foxc Function in Arterial Specification

During vascular development, angioblasts, which are multipotent endothelial progenitors originating from the mesoderm, coalesce and undergo vasculogenesis to form the primitive capillary plexus. Angiogenesis, the subsequent process of vascular remodeling, which gives rise to a mature network of blood vessels including arteries and veins, is regulated in part by hemodynamic forces. However, recent studies in zebrafish and mice clearly demonstrate that in the developing embryo, arterial and venous identity is established by genetic mechanisms before circulation begins.[42,43] For arterial specification (Fig. 1), vascular endothelial growth factor (VEGF) induces expression of Notch signaling genes, including *Notch1* and its ligand, *Delta-like 4* (*Dll4*) and also triggers a positive-feedback loop by inducing expression of *Neuropilin 1* (*Nrp1*), an arterial-specific coreceptor for VEGF. Upon activation of Notch signaling, the Notch effector genes, *Hey1/2* in mice or *gridlock* in zebrafish, further promote arterial differentiation. In contrast, the orphan nuclear receptor, COUP-TFII, is a determinant factor for venous specification by inhibiting expression of arterial specific genes, including *Nrp1* and *Notch/Dll4* (Fig. 1).[44]

Compound *Foxc1; Foxc2* homozygous mouse mutants show defective vascular remodeling of primitive blood vessels and abnormal vascular connections between arteries and veins (so called arteriovenous malformations).[8,10] Arteriovenous malformations similarly develop in endothelial

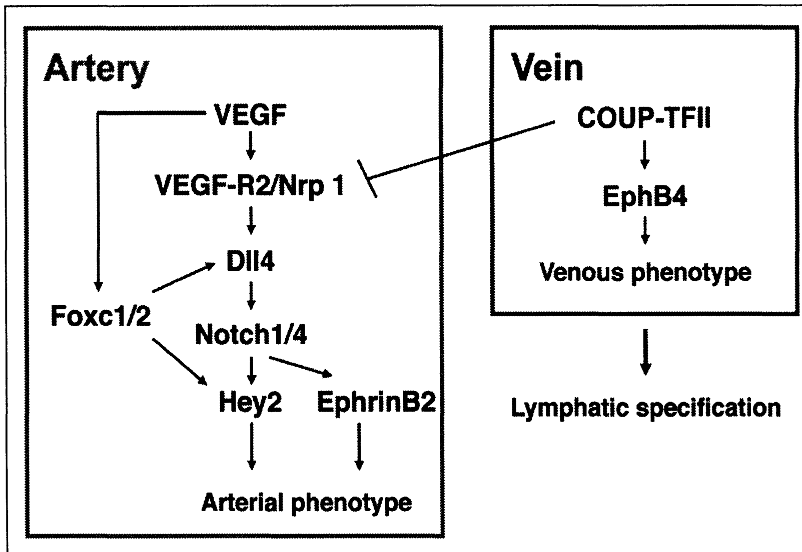

Figure 1. Genetic program of arterial-venous specification during vascular development. The VEGF and Notch pathways control the specification of arterial endothelial cells, while COUP-TF-II regulates venous cell fate. Foxc1 and Foxc2 interact with VEGF and Notch signaling and thereby induce arterial-specific genes, Dll4 and Hey2. Bi-directional Ephrin B2 and EphB4 signaling is induced arterial and venous endothelial cells, respectively and is involved in interactions between arteries and veins.

cells of mutant mice and zebrafish in which Notch signaling is defective.[45-49] Endothelial cells of compound *Foxc1; Foxc2* homozygous mutants fail to express arterial-specific genes such as *Nrp1* as well as Notch signaling molecules including *Notch1*, *Dll4* and *Hey2*, whereas venous markers such as COUP-TFII and EphB4 are normally expressed in compound homozygotes.[10] Most significantly, Foxc1 and Foxc2 can directly activate the *Dll4* promoter via a Foxc-binding element (FBE). Together, Foxc1 and Foxc2 act upstream of Notch signaling in arterial cell specification (Fig. 1).[10] This observation is consistent with the role of *Foxc* genes in regulation of Notch signaling events during the formation of the somites.[8]

In addition to *Dll4*, a recent study has demonstrated that Foxc1 and Foxc2 directly regulate expression of the Notch target gene, *Hey2* (also called *HRT2, HERP1, CHF1* and *Hesr–2*), by activating its promoter in endothelial cells.[25] Consistently, *Foxc*-mutant endothelial cells isolated from adult lungs of either endothelial-specific *Foxc1* mutant mice or *Foxc2*+/– mice show reduced expression of *Hey2*. The *Hey2* promoter includes two FBEs that are adjacent to a binding site for Suppressor of Hairless [Su (H)]. Upon activation of Notch signaling leading to a proteolytic cleavage to release the Notch intracellular domain (NICD) into the cytoplasm, Su(H) interacts with translocated NICD in the nucleus and is critical for Notch-mediated *Hey2* induction. When Foxc2 is combined with NICD, the *Hey2* promoter is synergistically activated as compared to either Foxc2 or NICD. In contrast, Foxc1 shows no synergistic effects on NICD-induced promoter activity. These data, together with the fact that Foxc2, but not Foxc1, directly binds to Su(H) and forms a protein complex with Su(H) and NICD, suggest that Foxc2 functionally interacts with Notch signaling to induce *Hey2* expression in endothelial cells.[25]

Foxc-induced promoter activity of *Dll4* and *Hey2* is significantly enhanced by VEGF in endothelial cells (Fig. 1).[25] In in vitro mammalian cell studies, the VEGF-mediated phosphoinositide 3-kinase (PI3K) pathway induces the transcription of *Notch1, Dll4* and *Hey2*.[25,50] Interestingly, modulation of Foxc activity by VEGF is enhanced by the PI3K pathway or inhibited by the extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) pathway. This

suggests that Foxc1 and Foxc2 interact with VEGF signaling in arterial gene expression. However, in the zebrafish embryo, the VEGF-activated PI3K pathway inhibits the stimulation of the ERK signaling cascade, leading to suppression of arterial differentiation.[51] Although reasons for the discrepancy between the in vitro and in vivo results remain unclear, one possible explanation is that these in vitro experiments were not conducted in uncommitted endothelial progenitor cells.[25,50] Since Foxc1 and Foxc2 are expressed in both arteries and veins in the mouse embryo,[10] it is possible that VEGF-mediated posttranslational modifications, such as phosphorylation, are critical for the activation of Foxc proteins in the induction of arterial-specific genes. Another interesting aspect of Foxc function in VEGF signaling is enhanced expression of *VEGF* in compound *Foxc1; Foxc2* mutants compared with the wild-type,[10] suggesting upregulation of a feedback response to impaired VEGF signaling.

There is now compelling evidence that arterial-venous cell fate determination is regulated by the multi-step regulatory system associated with the VEGF and Notch pathways.[42] A critical step is the induction of *Neuropilin 1* (*Nrp1*), coreceptor for VEGF and VEGF signaling promotes arterial differentiation as a positive feedback loop.[52] *Nrp1* expression is regulated by Foxc2 in endothelial cells.[25] Since COUP-TFII suppresses an arterial cell fate by inhibiting expression of Nrp1,[44] it will be important to determine whether Foxc proteins functionally counteract with COUP-TFII in the positive feedback loop of VEGF signaling during arterial-venous specification. These observations suggest that Foxc transcriptional factors control multiple steps of the VEGF-Notch/Dll4-Hey2 molecular cascade, thereby reinforcing arterial cell determination.

Foxc Function in Lymphatic Vessel Development

After arterial and venous endothelial cells differentiate, a subpopulation of venous endothelial cells is thought to become competent to acquire a lymphatic cell fate by progressively expressing the transcription factors Sox18 and Prox1 to differentiate into lymphatic endothelial cells.[53,54] The mammalian lymphatic vascular system originates solely from the venous endothelial cells.[55] VEGF-C, a VEGF receptor 3 (VEGFR-3) ligand, is expressed mainly in mesenchymal cells surrounding embryonic veins.[56] Prox1/VEGFR-3-positive lymphatic endothelial progenitors subsequently sprout from the veins via paracrine VEGF-C/VEGF-R3 signaling, leading to the formation of the lymphatic network, a process called (developmental) lymphangiogenesis.

Compound *Foxc1+/–; Foxc2–/–* mutant embryos show a significant reduction in the number of Prox1+ lymphatic endothelial cells sprouting from the cardinal vein (Fig. 2).[10] Importantly, *Sox18^{RaOP}* mutants have similar defects in lymphatic vessel formation and Sox18 can induce *Prox1* expression in the cardinal vein.[54] These data indicate that Sox18 directly acts upstream of Prox1 in the specification of lymphatic cell fate. Although *Foxc* genes and *Sox18* are co-expressed in lymphatic endothelial progenitors in the cardinal veins, the nature of functional interactions between Foxc proteins and Sox18 in lymphatic specification remains to be elucidated. On the other hand, expression domains of *Foxc1* and *Foxc2* overlap with those of *VEGF–C* in the mesenchyme surrounding the cardinal vein. Since compound *Foxc1+/–; Foxc2–/–* mutant embryos exhibit significant reduction in *VEGF-C* expression,[10] it is possible that Foxc1 and Foxc2 regulate the paracrine signal of VEGF-C in lymphatic vasculature development. This idea is supported by the finding that Foxc-dependent regulation of potent angiogenic factors, including Angiopoietin-2, in adipocytes influences vascular formation in a paracrine manner.[57]

Foxc Function in Angiogenesis

Angiogenesis is a critical process to grow new blood vessels from pre-existing vessels and involves endothelial cell proliferation, sprouting, migration and vascular tube formation. Angiogenesis is a necessary process in development, while pathological angiogenesis is involved in cancer and other ischemic diseases. Although angiogenic factors such as VEGF are known to control various processes of angiogenesis, the mechanistic basis for the regulation of endothelial gene expression is largely unknown. Recent studies have demonstrated that Foxc1 and Foxc2 control the process of angiogenesis by directly regulating the expression of two cell surface proteins in endothelial

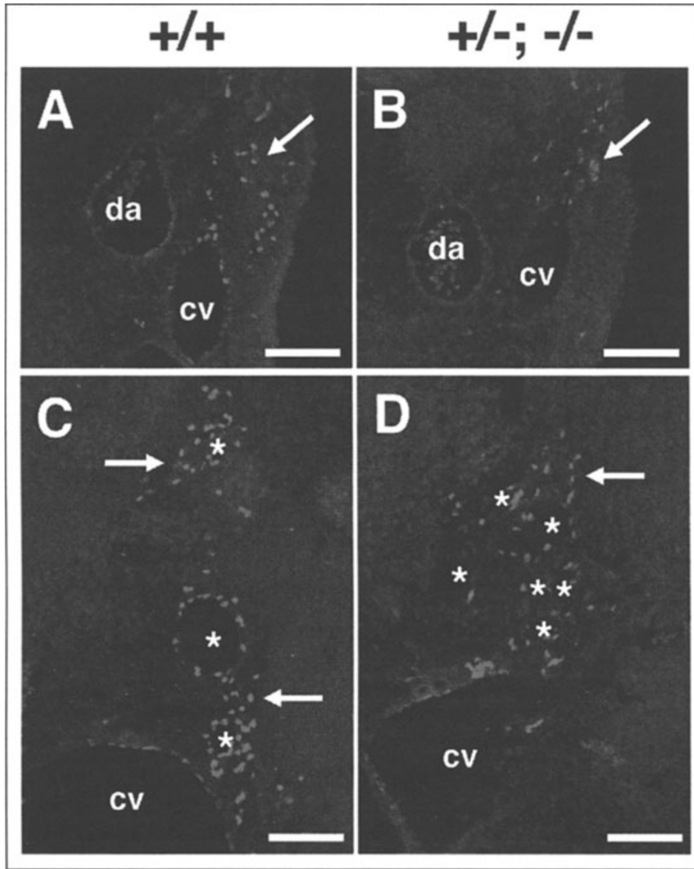

Figure 2. Compound *Foxc1+/-; Foxc2-/-* mutants have defective lymphatic vessel development. A-D) Immunohistochemical analysis to detect a lymphatic endothelial cell marker, Prox1, using transverse sections at the level of the heart at E10.5 (A,B) and E11.5 (C,D). A,B) Compound *Foxc1+/-; Foxc2-/-* mutant embryo (B) shows a reduction in the number of Prox1-positive lymphatic endothelial cells (arrows) from the cardinal vein (cv), compared to the wild type (A). C,D) At E11.5, the wild-type embryo (C) has well-formed lymph sacs (asterisks) and the sprouting of lymphatic endothelial cells (arrows). By contrast, abnormal formation of the lymph sacs and the reduced sprouting of lymphatic endothelial cells are observed in compound *Foxc1+/-; Foxc2-/-* mutant D. da, dorsal aorta. Scale bars, 50 μm. Adapted from Seo et al,[10] ©2006 with permission from Elsevier.

cells, the chemokine receptor CXCR4 and integrin β3,[24,30] which are essential for endothelial cell migration.[58,59] Upon binding of the CXCL12 ligand, CXCR4 activates downstream components to induce cell migration. The integrin β3 subunit forms a heterodimeric complex with the integrin αv subunit to allow interaction with extracellular matrix components. The integrin β3 subunit also functionally interacts with VEGF receptor 2 (VEGFR-2) in endothelial cells. Although Foxc2 does not enhance endothelial proliferation, Foxc2 increases endothelial cell migration, as well as sprouting and microvessel formation in aortic ring assay (Fig. 3).[30] In contrast, microvascular endothelial cells isolated from either endothelial-specific *Foxc1* mutant mice or *Foxc2+/−* mice show reduced cell migration.[24,30] These results indicate that Foxc transcription factors directly regulate angiogenesis via induction of integrin β3 and CXCR4.[60] Consistent with these findings,

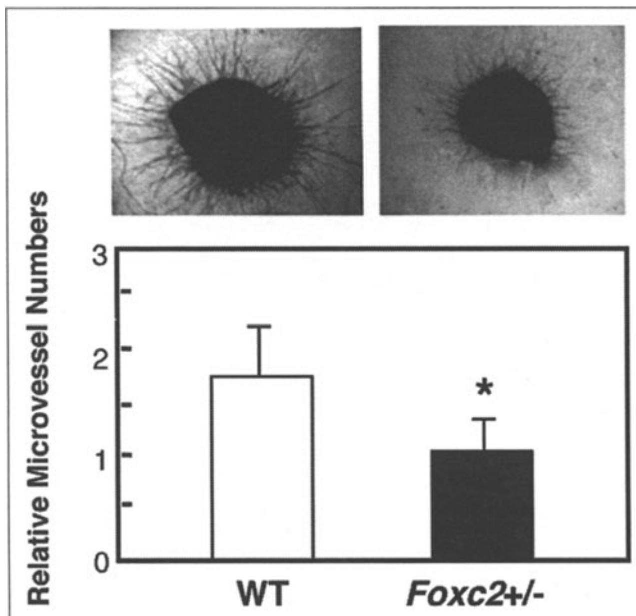

Figure 3. Foxc2 regulates angiogenesis. Aortic ring assay using adult aortas of wild-type and *Foxc2+/–* mice. Data are presented as the relative number of microvessels sprouting from aortic rings. Results are presented as the means ± S.D. (n = 9 or more). P values were determined by the corresponding sample indicated using Student's t test. *, $P < 0.05$ versus the corresponding control. Adapted from Hayashi et al,[30] ©2008 with permission from The American Society for Biological Chemistry.

Foxc2 has also been shown to enhance the migration of Maden-Darby canine kidney (MDCK) epithelial cells by upregulating matrix metalloproteinase (MMP)-2, 9.[61]

The Notch-Dll4 pathway is also critical for angiogenesis.[62] VEGF induces vascular sprouting through the filopodia of endothelial tip cells at the beginning of angiogenesis. On the other hand, Dll4 is induced in the tip cells by VEGF and precisely controls vessel branching. However, several important questions about this process need to be answered. For instance, it is currently unknown whether endothelial tip cells are positive for integrin $\alpha v\beta 3$ during vascular sprouting. Similarly, it remains to be elucidated whether the expression and activity of Foxc1 and Foxc2 are localized in the tip cells during angiogenesis.

Foxc Function in the Second Heart Field

The population of cardiac progenitors, derived from the anterior lateral mesoderm and located symmetrically in the cardiac crescent, is known as the first heart field (FHF) and gives rise to the left ventricle of the mammalian heart. The second heart field (SHF), which is originally located dorsal and medial to the FHF in the mesoderm, gives rise to cells that form the right ventricle, the outflow tract (OFT) and portions of the inflow tract.[63] The recent identification of genes involved in SHF progenitors, including *Islet 1* (*Isl1*), *Fox* genes (*Foxa2*, *Foxc1/c2* and *Foxh1*) and *Tbx1*, has provided the genetic and molecular basis for transcriptional regulation during the formation and development of the FHF and SHF.[63-66]

Although the FHF and SHF lineages derive from a common progenitor probably before the cardiac crescent stage (at the onset of gastrulation),[67] the two lineages diverge with distinct gene expression patterns. Whereas *Nkx2.5* and *Mef2c* are expressed in both lineages at the cardiac crescent stage, other genes appear to be restricted to either lineage. For instance, *Isl1* expression is

primarily detected in the SHF at the cardiac crescent stage and is downregulated as SHF progenitors migrate into the heart tube, although recent studies show that Isl1 is detected in both FHF and SHF regions at the cardiac crescent stage.[68] Together with the GATA4 transcription factor, Isl1 regulates the SHF-specific enhancer of *Mef2c*,[69] whereas activity of another SHF-specific enhancer of *Mef2c* requires the combination of Nkx2.5, Foxh1 and Smad.[70] Like *Mef2c* expression in the SHF, Isl1 can also cooperate with GATA4 as well as Tbx20 to activate the SHF enhancer of *Nkx2.5*.[71] *Isl1* mutant embryos lack the OFT and right ventricle and Isl1 is required for the proliferation and survival of SHF cells as well as transcriptional regulation of other SHF genes and signaling molecules such as bone morphogenetic proteins (BMP) and fibroblast growth factors (FGF).[72] Therefore, Isl1 is a key early regulator for the molecular hierarchy in SHF progenitors. Another key molecule is *Tbx1*, which is lost in 22q11.2 deletion syndrome in humans and is required for the alignment and separation of the OFT.[73] Of note, Fox transcription factors (Foxa2, Foxc1 and Foxc2) have been shown to activate an enhancer sufficient to drive *Tbx1* expression in the SHF,[74] while Tbx1, in turn, regulates an enhancer of *Fgf8* in the pharyngeal mesoderm.[75]

Compound *Foxc1; Foxc2* mutant embryos have a wide spectrum of early cardiac abnormalities. These include hypoplasia or lack of the OFT, right ventricle and the inflow tract as well as abnormal formation of the epicardium in a dose dependent manner (Fig. 4).[11] In SHP progenitors and their derivatives in compound *Foxc1; Foxc2* mutants, expression of *Tbx1* and *Fgf8/10* is significantly

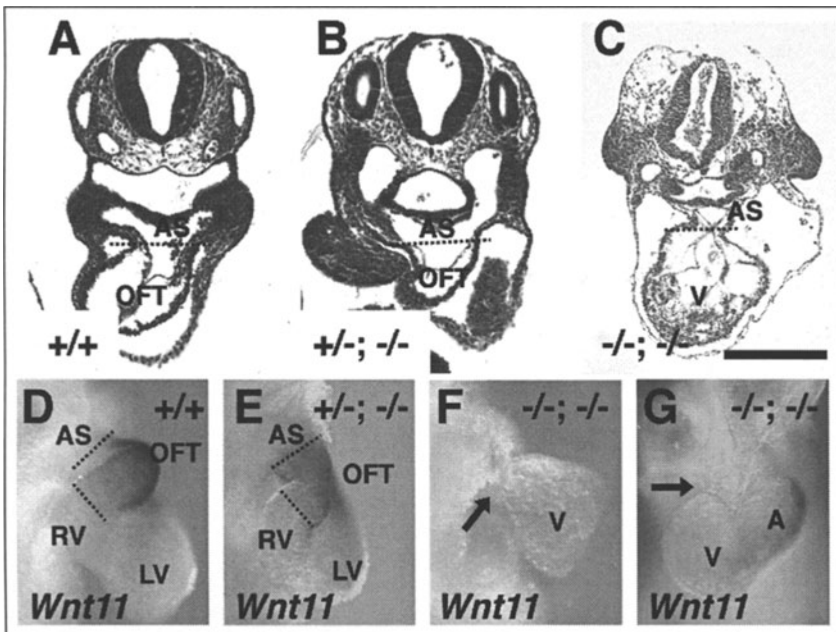

Figure 4. Compound *Foxc1; Foxc2* mutant embryos have cardiac abnormalities. A-C) Histological analysis of wild-type (A) and compound *Foxc1+/–; Foxc2–/–* (B) and *Foxc1–/–; Foxc2–/–* mutant (C) embryos at E9.0 at the levels of the heart. Compound *Foxc1; Foxc2* mutant embryos show hypoplasia (B) or lack (C) of the OFT in a dose-dependent manner. Note the direct connection of the disorganized aortic sac (AS) to the ventricle (V) in compound *Foxc1–/–; Foxc2–/–* mutant (C). Dotted lines indicate the boundary between the aortic sac (AS) and outflow tract (OFT). D-G) Whole-mount in situ hybridization at E9.0 to detect *Wnt11* expression in the OFT. The expression domain of *Wnt11* is reduced in compound *Foxc1+/–; Foxc2–/–* mutant (E, right view), while it is not detected in compound *Foxc1–/–; Foxc2–/–* mutant (arrows) (F, right view; G, front view). Dotted lines demarcate the expression domain of *Wnt11* in the OFT. A, atrium; LV, left ventricle; RV, right ventricle; V, ventricle. Scale bar, 100 μm. Adapted from Seo and Kume,[11] ©2006 with permission from Elsevier.

downregulated, whereas *Isl1* expression is slightly reduced but still remains. This observation suggests that Foxc1 and Foxc2 function upstream of the Tbx1-FGF cascade during the morphogenesis of the OFT. Since it is unknown whether Foxc proteins and Isl1 functionally interact with each other in SHP progenitors, it remains to be determined whether a reduction in cell proliferation in the SHF of compound *Foxc1; Foxc2* mutants is due to a failure of the expansion of the Isl1-positive SHP lineage. Foxc1 and Foxc2 can directly regulate *Tbx1* expression in the SHF as well as head mesenchyme by binding to multiple FBEs on its enhancers.[74,76] Since compound *Foxc1; Foxc2* mutants have much more severe OFT defects than *Tbx1* mutants, it is plausible that in addition to controlling *Tbx1* expression, Foxc1 and Foxc2 are required for regulating additional genes/pathways in SHF development.

Foxc Function in Cardiac Neural Crest Cells

Cardiac neural crest cells are a nonmesodermally derived cell population that significantly contributes to the developing heart. They arise from the caudal hindbrain (rhombomeres 6-8), migrate through the branchial arch 3, 4 and 6 and invade the aortic arch and OFT of the heart.[77] This cell population subsequently participates in OFT septation and differentiates into the cardiac ganglia and the tunica media of the great vessels. Disruption of the cardiac neural crest before migration in chick embryos leads to a variety of malformations such as interruption or coarctation of the aortic arch, VSD and persistent truncus arteriosus (PTA).[77,78] Ablation of the cardiac neural crest is also associated with aplastic or hypoplastic thymus, parathyroid and thyroid glands. These abnormalities are frequently seen in human congenital syndromes, including DiGeorge syndrome, which in most cases results from chromosome 22q11.2 deletion (*del22q11*). Moreover, cardiac neural crest cells influence cardiac development in a non-cell autonomous manner. Ablation of the cardiac neural crest in the chick results in a failure to elongate the SHF-derived OFT myocardium.[79] Although the precise effects of the cardiac neural crest on SHF development remain unknown, this cell population alters the availability of FGF8 in the caudal pharynx.[80,81]

Besides the broad expression of *Foxc1* and *Foxc2* in the mesoderm and it derivatives, the two genes are also expressed in the neural crest cell lineage. As described above, single *Foxc* mutant mice exhibit abnormal aortic arch patterning, suggesting that Foxc1 and Foxc2 in the neural crest play a role in remodeling aortic arch arteries.[5,13] Furthermore, cardiac neural crest cells of compound *Foxc1; Foxc2* mutant embryos undergo abnormal apoptosis during migration, leading to a failure of the OFT septation (PTA), a characteristic phenotype of the ablation of this cell lineage in the chick.[11] This observation indicates that Foxc1 and Foxc2 are required for the survival for cardiac neural crest cells. Despite extensive apoptosis, *Foxc*-mutant cardiac neural crest cells are able to differentiate into smooth muscle. Compound *Foxc1+/–; Foxc2–/–* mutants also show lack of the 2nd pharyngeal arch, suggesting defects in cranial neural crest cells.[8] Although the indirect effects of cardiac neural crest cells on the addition of SHF-derived myocardial cells to the developing heart have been suggested,[78,82] autocrine FGF signaling in the SHF is primarily required for OFT morphogenesis.[83] Thus, given the fact that reduced expression of SHF markers such as *Tbx1* and *Fgf8/10* is already observed in compound *Foxc1; Foxc2* mutant embryos at E8.5 before the onset of cardiac neural crest migration, the OFT abnormalities in these *Foxc* mutants are likely attributable to defective SHF progenitors in a cell-autonomous manner.

Foxc Function in Epicardial-Derived Cells

Another tissue that is critical to form the heart is the epicardium, which is the epithelial cell layer that covers the surface of the heart. The epicardium originates from a specific population of mesothelial cells from the proepicardium (PE). The epicardium plays an essential role in coronary vessel development by providing a source of vascular smooth muscle and endothelial cells after epithelial to mesenchymal transformation (EMT).[84,85] Importantly, multipotent epicardial progenitor cells that differentiate into cardiac myocytes have recently been identified.[86,87]

Both *Foxc1* and *Foxc2* are expressed in a subset of cells in the PE before the formation of the epicardium, while transcripts of the *Foxc* genes appear to be downregulated in the developing epicardium.[11] Compound *Foxc1+/−; Foxc2−/−* mutant embryos have abnormal formation of the epicardium, which is detached from the underlying myocardium, while *Foxc* single mutants or compound *Foxc1−/−; Foxc2+/−* mutants normally form the epicardium. Although the epicardial-derived mesenchymal cells are generated over the entire surface of the heart through EMT, the majority of these cells are produced at the atrioventricular junction.[88] Intriguingly, compound *Foxc1+/−; Foxc2−/−* mutants show abnormal accumulation of mesenchymal cells that are particularly localized at the subepicardial space of the conoventricular region and some of these cells are differentiated into either smooth muscle or endothelial lineage.[11] It is, however, unclear whether the mesenchymal cells at the subepicardial space of compound *Foxc1; Foxc2* mutants are solely derived from the epicardium.

Signal(s) from the myocardium are important for the initiation of EMT in the epicardium[89] and several molecular signals such as VEGF, FGF and TGFβ are critical for the regulation of epicardial EMT.[84,90] Since compound *Foxc1+/−; Foxc2−/−* mutants have the above-mentioned defects in the OFT, the abnormal mesenchymal phenotype at the subepicardial space may result from dysregulation of myocardially-derived signaling molecules. Another, nonexclusive possibility is that compound *Foxc1; Foxc2* mutants may lack proper cell-cell interactions between the myocardium and epicardium. Further experiments are needed to clarify the nature of epicardial defects in compound *Foxc1; Foxc2* mutants.

Future Directions

I have presented a summary of the current understanding of the cooperative roles of Foxc1 and Foxc2 in cardiovascular development. Evidently, they are key transcriptional regulators controlling multiple processes in this system. However, many important questions about the function of the *Foxc* genes in cardiovascular development remain to be answered. At present, much of our knowledge about Foxc function has been obtained from the studies of conventional *Foxc* mutant mice. Given their broad expression in mesodermal and neural crest derivatives, tissue- and/or time-specific ablation of *Foxc* genes will provide further invaluable information on the direct involvement of *Foxc* genes in the development of the cardiovascular system. For example, it has recently been shown that cardiac neural crest cells and SHF-derived cells reciprocally interact with each other during OFT morphogenesis.[83] In addition, although the two *Foxc* genes have overlapping expression patterns in the cardiovascular system, an unsolved question is whether they have similar but distinct functions. It is important to note that Foxc2, but not Foxc1, can functionally interact with Notch-mediated transcription in endothelial cells.[25] Moreover, given evidence that other *Fox* genes are also expressed during cardiovascular development, functional redundancy of the *Fox* gene family must be considered. For instance, Foxh1 is essential for the development of the SHF,[70] while Foxo1 mutant embryos have impaired angiogenesis.[91,92] A new aspect of the mechanism of Foxc function in vascular development is the combinatorial activity of Foxc2 and the Ets transcription factor Etv2 in regulating endothelial-specific gene expression during early development.[93] Further studies are needed to reveal the mechanisms of transcriptional regulation involving Foxc and other transcription factors/cofactors.

Although knockout and transgenic approaches in mice and other species have facilitated cardiovascular research over the past decade, the utility of stem cell-based research on cardiac and endothelial cell differentiation is likely to lead to significant progress in deciphering complex networks of transcriptional events associated with multiple signaling pathways. In particular, recent studies have shown that signaling pathways such as FGF, BMP and Wnt are critical for the induction and expansion of cardiac progenitor cells.[94] Therefore, it is anticipated that future studies using cell-based approaches will contribute to understanding the molecular mechanisms that control the genetic program associated with the critical pathways and Foxc-mediated transcriptional regulation in cardiovascular progenitors.

Acknowledgements

Research in the author's laboratory on the role of Foxc transcription factors during cardiovascular development was supported by the National Institutes of Health, American Heart Association and March of Dimes Foundation. The author would like to thank Drs David Bader and Joey Barnet for insightful discussion.

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