Roles of forkhead transcription factor Foxc2 (MFH-1) and endothelin receptor A in cardiovascular morphogenesis

Naoko Kanzaki-Kato\textsuperscript{a,b}, Tomoki Tamakoshi\textsuperscript{c}, Yan Fu\textsuperscript{a}, Abhishek Chandra\textsuperscript{c}, Tatsuo Itakura\textsuperscript{c}, Tadayoshi Uezato\textsuperscript{c}, Toshinobu Tanaka\textsuperscript{b}, David E. Clouthier\textsuperscript{d}, Toshihiro Sugiyama\textsuperscript{a}, Masashi Yanagisawa\textsuperscript{d}, Naoyuki Miura\textsuperscript{c,*}

\textsuperscript{a}Department of Biochemistry, Akita University School of Medicine, 1-1-1 Hondo, Akita 010-8543, Japan
\textsuperscript{b}Department of Gynecology and Obstetrics, Akita University School of Medicine, 1-1-1 Hondo, Akita 010-8543, Japan
\textsuperscript{c}Department of Biochemistry, Hamamatsu University School of Medicine, 1-20-1 Handa-yama, Hamamatsu 431-3192, Japan
\textsuperscript{d}Department of Molecular Genetics, and Howard Hughes Medical Institute, University of Texas Southwestern Medical Center, Dallas, TX 75390-9050, USA

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Abstract

\textbf{Objective:} Foxc2/MFH-1 is a member of the forkhead family of transcription factors and Foxc2-deficient mice exhibit aortic arch anomalies (type B interruption of the aortic arch). Endothelin receptor type-A (ETA) is one of the two known endothelin receptors that belong to the G-protein-coupled receptor family. ETA-deficient mice show defects in the great arteries, primarily type B interruption of the aortic arch. Based on similar phenotypes in the cardiovascular system of Foxc2- and ETA-deficient mice, we investigated whether Foxc2 and ETA have a close relationship in aortic arch patterning.

\textbf{Methods:} The Foxc2 and ETA homozygotes were obtained by crossing the Foxc2 and ETA heterozygotes, respectively. The double Foxc2/ETA homozygotes were obtained by crossing the double Foxc2/ETA heterozygotes.

\textbf{Results:} We investigated the expression of ETA in Foxc2-null mice and the expression of Foxc2 in ETA-null mice and found that the absence of either Foxc2 or ETA had no effect on the expression of the other. Next, we analyzed mice lacking both Foxc2 and ETA to examine the relationship between Foxc2 and ETA on aortic arch patterning in vivo. We found that the majority of Foxc2/ETA double-mutant embryos died around 11.5 dpc and that all surviving mice had persistent truncus arteriosus.

\textbf{Conclusions:} The results suggest that Foxc2- and ETA-expressing cells additively form the aorticopulmonary septum.

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\textbf{Keywords:} Foxc2; Arteries; Developmental biology; Gene expression; Heart failure

1. Introduction

The aortic arch is formed by a complicated series of morphogenic events. During embryogenesis, five pairs of pharyngeal arch arteries appear in rostral to caudal direction, following the generation of the dorsal aorta. Arch arteries arise from the aortic sac and terminate in the dorsal aorta, traversing the corresponding pharyngeal arches. Arch arteries 1 and 2 regress and become capillaries in pharyngeal arches 1 and 2 by E 11.0, whereas arch arteries 3 and 4 continue to enlarge. Bilateral pulmonary arteries branch off from the proximal part of arch artery 6. The arch arteries 3, 4 and 6 develop symmetrically until around E 11.5. However, arch arteries undergo dramatic remodeling to correctly establish the embryonic circulatory system and to prepare for postnatal circulation [1,2].

The molecular mechanisms of arch artery remodeling and genetic determinants are not yet fully understood. The
intimate involvement of cardiac neural crest cells in this process has been suggested by the phenotype resulting from ablation of the cardiac neural crest in chick embryos, where various types of aortic abnormalities and septation defects of the outflow tract develop [3]. The cardiac neural crest derives from the dorsal aspect of the neural tube at a level between the otic placode and somite 3. Cardiac neural crest cells undergo epithelial–mesenchymal transformation and migrate through branchial arches 3, 4 and 6, and the circumpharyngeal region, eventually to the outflow tract of the heart [4]. It is shown in quail-chick chimeras that the mesenchyme of branchial arch arteries is mostly made from neural crest cells [5]. The contribution of cardiac neural crest cells to the formation of the aortopulmonary septum is also demonstrated in chick embryos with ablated neural crest [6]. Certain types of human congenital cardiovascular malformations, including type B interruption of the aortic arch (IAA) [7], persistent truncus arteriosus (PTA) [8] and the DiGeorge syndrome [9,10] strongly resemble defects seen in embryos with neural crest ablations. However, the precise cellular and molecular mechanisms by which the neural crest is involved in cardiovascular development are not completely understood.

Mesenchyme forkhead-1 (MFH-1)/Foxc2 is a member of the forkhead family of transcription factors that are strongly expressed in mesoderm tissues in mouse embryos. Foxc2-deficient mice exhibit aortic arch anomalies and defects of skeletogenesis in the craniofacial bones and vertebral column. This indicates that Foxc2 plays important roles in aortic arch remodeling and skeletogenesis [11–15]. The most common aortic malformation in Foxc2-null mice is the interruption of the aortic arch between the common carotid artery and left subclavian artery (type B interruption of the aortic arch).

Endothelin-1 (ET-1) is a 21-amino-acid peptide with various biological activities including vasoconstriction and cell proliferation, and was first identified in the medium of cultured vascular endothelial cells [16]. Three isopeptides (ET-1, 2, and 3) encoded by different gene loci constitute its gene family [17]. Endothelin receptor type-A (ETA) is one of the two known endothelin receptors that belong to the G-protein-coupled receptor family. It binds ET-1 and ET-2, but not ET-3 at physiological concentrations [18]. ETA-deficient mice show defects in craniofacial structures, the great arteries and the cardiac outflow tract as seen in ET-1-null mice [19–22]. The most common malformation of the great artery in ETA-null mice is the type B interruption of the aortic arch, just as in Foxc2-null mice. The similarity of this phenotype in the cardiovascular system of Foxc2- and ETA-deficient mice suggests that Foxc2 and ETA are closely linked in aortic arch morphogenesis. In this study, we investigated the expression of ETA in Foxc2-null mice and the expression of Foxc2 in ETA-null mice. The results of in situ hybridization and RT-PCR analysis showed that the absence of either Foxc2 or ETA had no effect on the expression of the other. Next, in order to examine the relationship between Foxc2 and ETA on aortic arch patterning in vivo, we analyzed mice lacking both Foxc2 and ETA. We found that the majority of Foxc2/ETA double-mutant embryos died due to heart failure around 11.5 dpc.

2. Methods

2.1. Mice

MFH-1/Foxc2 mutants (Foxc2(tm1Miu) [13]) were crossed with the C57BL/6 mice and maintained. ETA mutants [21] were bred with C57BL/6 mice. Foxc2/ETA double heterozygotes were generated by crossing Foxc2 heterozygotes and ETA heterozygotes. The offspring from Foxc2/ETA double heterozygote matings were genotyped by polymerase chain reaction (PCR) analysis of tail DNA and by Southern blot analysis of yolk sac DNA. The oligonucleotides and probes used to genotype Foxc2 mutants and ETA mutants were previously described [13,21].

2.2. Histology and in situ hybridization

Mice were mated overnight and females were examined for a vaginal plug on the following morning. Noon on the day of evidence of a vaginal plug was considered 0.5 dpc. Embryos were fixed in 10% formalin or phosphate-buffered 4% paraformaldehyde overnight, dehydrated, embedded in Paraplast and sectioned at 7 μm. Sections were stained with hematoxylin and eosin. In situ hybridization on tissue sections was performed as described [23] by using cRNA transcribed from the NotI–EcoRI fragment of Foxc2 cDNA [11] and the BamHI–EcoRI fragment of ETA cDNA [21] as probes.

2.3. RT-PCR

The third, fourth and sixth pharyngeal arches of 11.5 dpc embryos were excised and total RNA was isolated from the excised part of embryos using RNeasy (Quiagen, K.K.),
Tokyo, Japan). First-strand cDNA synthesis was performed using 5 µg of total RNA with Superscript II and oligo(dT)12–18 primer, according to the manufacturer’s instructions (Invitrogen, Carlsbad, CA). The resulting template was subjected to a PCR reaction using the following primers: 

- Foxc2, 5′-GCCACCTCCTGTGATCT-GAAC-3′ and 5′-GGACGCTCAGTTTGTG-3′;
- ETA, 5′-CATGAACTAACCCCTCGCAG-3′ and 5′-GAAATGACATGCCGCTATGC-3′;
- EF-1, 5′-CGAGGCAATGTGGCTGGTGAC-3′ and 5′-TCTGGCTCATTG-CAGA-3′.

PCR was carried out by denaturing the DNA at 94 °C for 1 min, followed by 35 (Foxc2), 38 (ETA) and 15 (EF-1) cycles at 94 °C for 1 min, 57 °C for 1 min, 72 °C for 1 min, and a final extension step at 72 °C for 10 min.

2.4. India ink injection

Foxc2/ETA double heterozygotes and Foxc2/ETA double homozygotes were dissected from the decidua at 10.5 dpc and India ink (Carbon Black; Dainihon Ink chemicals, Suita, Japan) was injected directly into the beating heart. Embryos were fixed in 95% ethanol, 5% chloroform and 1% acetic acid overnight and cleared in a 1:1 solution of methyl salicylate/benzyl benzoate [24].

3. Results

3.1. The expression of Foxc2 and ETA in wild-type and mutant embryos

We examined the expression of Foxc2 and ETA in 9.5, 10.5 and 11.5 dpc wild-type mouse embryos. In 9.5 dpc embryos, strong Foxc2 expression was detected in the mesenchyme of the head and the pharyngeal arches and dorsal aortas, and in presomitic mesoderm, with weak expression detected in the heart and its outflow tract (data not shown). ETA expression was detected ubiquitously in the mesenchyme of the pharyngeal arches, and in head and body mesenchyme, the heart and the outflow tract (data not shown). Neither Foxc2 nor ETA expression was detected in the neural tube. In 10.5 and 11.5 dpc embryos, Foxc2 expression was strongly detected in the mesenchyme of the third, fourth and sixth arch arteries on the right and left sides as well as in the dorsal aortas (Fig. 1C,K and data not shown). ETA expression was diffusely detected in body mesenchyme including the mesenchyme of the third, fourth and sixth arch arteries at the same time in mouse embryos although their expression patterns were different (Fig. 1C,D).

Since Foxc2-deficient mice and ETA-deficient mice frequently show type B interruption of the aortic arch [13,21] and Foxc2 and ETA are expressed in the pharyngeal arch at the same time in mouse embryos, a close relationship was suggested between Foxc2 and ETA. We first investigated the expression of ETA mRNA, especially in the area of the developing arch artery in Foxc2-null embryos (Fig. 1F,H). In situ hybridization sections showed that ETA was diffusely expressed in the mesenchyme of the pharyngeal arches at 10.5 and 11.5 dpc in Foxc2-null embryos (Fig. 1H and data not shown), which was the same pattern as in wild-type embryos (Fig. 1G). Next, we investigated the expression of Foxc2 in the ETA-null mice. In situ hybridization sections showed that Foxc2 was strongly expressed in the mesenchyme of the third, fourth and sixth arch arteries and in the dorsal aortas on both sides in 10.5 and 11.5 dpc ETA-null embryos (Fig. 1L and data not shown) as well as in wild-type embryos (Fig. 1K).

Next we did semiquantitative RT-PCR to compare the expression of Foxc2 and ETA mRNA in the 11.5 dpc knockout mice. Fig. 2 showed that the Foxc2 and ETA mRNAs were not down-regulated nor up-regulated in the ETA-null and Foxc2-null mice, respectively. We concluded that Foxc2 and ETA are expressed independently.

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**Table 1**

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<th>Foxc2 genotype</th>
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**Fig. 2.** RT-PCR analysis of the Foxc2 and ETA mRNAs in the wild-type and knockout mice. Total RNAs (5 µg) from the third, fourth and sixth pharyngeal arches of 11.5 dpc wild-type and knockout mice were reverse-transcribed. Two samples of cDNAs from Foxc2−/−, wild-type and ETA−/− mice were subject to quantitative PCR for the Foxc2, ETA and elongation factor-1 (EF-1) mRNA and the PCR products were separated in 6% polyacrylamide gel electrophoresis.
3.2. All Foxc2/ETA double mutant embryos die in utero

To examine the relationship between Foxc2 and ETA on aortic arch formation in vivo, we created double mutants. Double heterozygous Foxc2 and ETA mice were healthy and fertile, and the cardiovascular phenotypes were not affected. Double heterozygotes were intercrossed to generate double homozygotes. Genotyping of 123 offspring obtained by near-term Cesarean section from heterozygous intercrosses revealed no homozygous Foxc2/ETA double mutants (Table 1), suggesting embryonic lethality. In Table 1, the numbers of Foxc2+/C0/C0 mice (16/123; 13%) and ETA+/C0/C0 mice (39/123; 32%) were almost the same as the expected frequency, which was reported previously [13,21].

To define the time and cause of death of Foxc2/ETA double mutants, we performed genotyping in earlier embryos. At 10.5 dpc, the number of double homozygous mice (6/145; 4.1%) obtained from double heterozygous crosses was near the expected percentage (6.5%), indicating that double homozygous embryos were viable up to 10.5 dpc (Table 2). At 11.5 and 12.5 dpc, the frequencies of viable double homozygous mutant mice were 2/208 (1%) and 3/295 (1%), respectively. These results suggest that the majority of Foxc2/ETA double-mutant embryos died around 11.5 dpc.

3.3. Cardiovascular phenotypes in Foxc2/ETA double-mutant embryos

At 10.5 dpc, wild-type and homozygous Foxc2/ETA double-mutant embryos were indistinguishable grossly.

![Fig. 3. Signs of heart failure were detected in double homozygotes. Foxc2/ETA double heterozygotes (A–C) and double homozygotes (D–F) at 10.5 dpc (C, F) and 11.5 dpc (A, B, D, E) were fixed and the sections were stained with hematoxylin and eosin. Frontal section. Note that the pericardial space (asterisks in E and F) was enlarged and the atrium was dilated (“at” in D).](#)

![Fig. 4. Arch arteries and dorsal aortas in Foxc2/ETA double homozygotes. India ink was injected into the beating hearts of the Foxc2/ETA double heterozygotes (left panels) and double homozygotes (right panels) at 10.5 dpc, and the embryos were fixed and cleared. Upper panels: view from the right side. Lower panels: view from the left side. Note that the dorsal aortas between the third and fourth arch arteries were closed on both sides (ductus caroticus; blue arrowheads) and that the dorsal aorta between the second and third arch arteries was obstructed on the left side (red arrowhead). III, IV, VI and D indicate the third, fourth and sixth arch arteries and dorsal aortas, respectively.](#)
Fig. 5. Comparison of the outflow tract of the heart. Foxc2/ETA double heterozygous (A) and double homozygous embryos (B) at 12.5 dpc were fixed and the sections were stained with hematoxylin and eosin. Frontal section. Note that the aorticopulmonary septum (AP) was not formed in Foxc2−/− ETA−/− embryos. AS, ascending aorta; PT, pulmonary trunk; TA, truncus arteriosus. Magnifications: ×100.

Four double homozygous 10.5 dpc mutants were histologically examined. No remarkable abnormalities were detected in the muscle and trabeculation in the heart (data not shown). However, the pericardial space was enlarged in double homozygotes (Fig. 3F), but not in double heterozygotes (Fig. 3C) and single homozygotes (data not shown). To analyze the vascular structure of the branchial arch arteries, we injected India ink into the beating heart of 10.5 dpc embryos (Fig. 4). The third, fourth and sixth arch arteries were formed symmetrically linking the aortic sac and dorsal aortas in double homozygous and double heterozygous mutants. Interestingly, the dorsal aortas between the second and third arch arteries, and between the third and fourth arch arteries (the ductus caroticus) were closed in double homozygotes (red and blue arrowheads, respectively in Fig. 4, right panels). Between 11.0 and 13.0 dpc, the aortic sac and outflow tract are divided longitudinally by the aorticopulmonary septum (AP, Fig. 5A) [25,26], thus giving rise to the ascending aorta (AS, Fig. 5A) and pulmonary trunk (PT, Fig. 5A). In two 11.5 dpc and three 12.5 dpc double homozygous Foxc2/ETA mutants, no sign of aorticopulmonary septation was observed (Fig. 5B and data not shown), whereas the aorticopulmonary septum was formed in Foxc2/ETA double heterozygous embryos (Fig. 5A and data not shown) and Foxc2 and ETA single homozygous embryos (data not shown). In these double homozygotes, the atrium was dilated (Fig. 3D) and the pericardial space was enlarged (Fig. 3E). In double heterozygotes and Foxc2 and ETA single homozygotes, these abnormalities were not detected (Fig. 3A,B and data not shown). These results suggest that heart failure might occur and aorticopulmonary septation might be impaired in double homozygotes.

4. Discussion

4.1. No upstream/downstream relationship between Foxc2 and ETA genes

We have shown that Foxc2 is essential for the normal development of aortic arch remodeling [13]. The cardiovascular phenotype suggests that Foxc2 plays a role in the migration, proliferation, and/or differentiation of cardiac neural crest cells. We and Kurihara have shown that ET-1/ETA-mediated signaling is essential for the normal development of the great arteries and cardiac outflow structures [19–21,27].

In this study, we first examined whether Foxc2 and ETA affected the aortic arch remodeling in a way that depends on cardiac neural crest cells. In 10.5 and 11.5 dpc wild-type mouse embryos, both Foxc2 mRNA and ETA mRNA are distinctly expressed in the mesenchyme of the arch arteries, which are derived from postmigratory neural crest cells as previously reported [13,27,21]. In addition, we could not detect a change in the expression levels of Foxc2 and ETA genes in the ETA-null and Foxc2-null mice, respectively. These results indicate that Foxc2 and ETA are expressed independently.

4.2. Foxc2/ETA double-mutant embryos succumb to mid-embryonic lethality

Next, we examined the relationship between Foxc2 and ETA in vivo by analyzing the phenotype of Foxc2/ETA double mutants. From the point of intrauterine viability, half of the Foxc2-null embryos died in utero around 12.5 dpc, and the other half of the embryos died within 15 min after birth. Cardiovascular defects, including interruptions and ventricular septal defect (VSD), were frequently present. In Foxc2-null mice, the development of arch arteries is normal at 11.5 dpc, and the left fourth arch artery regresses during remodeling of the aortic arch around 12.5 dpc [13]. All ETA-null mice were born and died soon after birth. The cardiovascular defects in ETA-null mice include interruption of the aortic arch, VSD, and persistence of some vessels that normally regress, as reported previously ([21,27], and data not shown). These results indicate that the phenotypes of Foxc2/ETA double homozygotes were more severe than those of single homozygotes.

In this study, we showed that the majority of Foxc2/ETA double-mutant embryos died around 11.5 dpc. All five exceptional surviving double-mutant embryos obtained at 11.5 and 12.5 dpc showed PTA without VSD nor IAA. This means that aorticopulmonary septation was impaired in the double-mutant embryos. India ink injection analysis showed that arch arteries 3, 4 and 6 were formed symmetrically, linking the aortic sac and dorsal aortas at 10.5 dpc (Fig. 4). Histological examination indicates that double homozygotes showed signs of heart failure, pericardial edema and atrial dilatation (Fig. 3). Nearly all double-mutant embryos were dead by 11.5 dpc, the time during aortic arch formation that arch arteries 3, 4 and 6 undergo dramatic remodeling to correctly establish the embryonic circulatory system [1,2]. We speculate that double homozygotes that had PTA, IAA and VSD were dead at 10.5 dpc or just after 10.5 dpc and that the small number of double homozygotes that had PTA but not VSD nor IAA could be alive until 11.5 and 12.5 dpc.
In the double-mutant embryos, the dorsal aortas between the second and fourth arch arteries were obstructed in one embryo (Fig. 4). In the other four double homozygotes, the dorsal aortas between the second and third arch arteries were narrowed (data not shown). However, it is considered that the dorsal aortas are not derived from the neural crest [28]. These would suggest either that double deletions of Foxc2/ETA affect non-neural crest-derived outflow arteries or that the abnormalities observed in Indian ink injection may occur secondarily to heart failure.

4.3. Roles of Foxc2 and ETA genes in cardiac neural crest cells

The morphogenesis of the outflow tract and the aortic arch arteries is initiated by mesenchymal cells derived from the cardiac neural crest, which migrate between the vascular endothelium and the pharyngeal mesoderm from the pharyngeal arches and thereby envelop the vessel [29]. The similar cardiovascular phenotypes of Foxc2 and ETA single homozygotes suggest that both Foxc2 and ETA play important roles in cardiac neural crest development [13,21,27]. In this study, we showed that the third, fourth and sixth arch arteries were formed almost normally up to 10.5 dpc without Foxc2 and ETA (Fig. 4). It seems that the cardiac neural crest is not essential for the early formation of the aortic arch arteries, but that once the arteries have formed and are undergoing transformation to the adult phenotype, the neural crest becomes critical to the maintenance of a major arch artery [29].

In the chick embryo, PTA was found only after ablation of the neural crest contributing to arches 3, 4, and 6, singly or in combination. A remarkably higher incidence (72.7%) of PTA was found with large ablations of neural crest from arch 4 and 6 areas [30], which means that more neural crest cells are necessary for aorticopulmonary septation. In this study, we showed that the surviving embryos of Foxc2/ETA double homozygotes had PTA (Fig. 5B), while Foxc2 and ETA single homozygotes rarely had PTA. We speculate that Foxc2 and ETA act distinctly on cardiac neural crest cells contributing to arches 3, 4 and 6; some populations are overlapping and other populations are not overlapping. A small loss of cardiac neural crest cells may induce type B interruption of the aortic arch as seen in Foxc2 and ETA single homozygotes. A larger loss of cardiac neural crest cells in Foxc2/ETA double mutants may lead to impairment of septum formation, or PTA.

5. Conclusion

In this study, we could not get a direct evidence of an upstream/downstream relationship between Foxc2 and ETA. Double Foxc2/ETA homozygotes died earlier than single homozygotes due to heart failure. Interestingly, double homozygotes showed PTA. These results suggest that Foxc2 and ETA additively form an aorticopulmonary septum.

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