Partial rescue of the Na⁺-Ca²⁺ exchanger (NCX1) knock-out mouse by transgenic expression of NCX1

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Accepted 18 April 2003

Abbreviations: ED, embryonic day; H & E, hematoxylin and eosin; KO, knock-out; NCX1, Na⁺-Ca²⁺ exchanger 1; RT, reverse transcription; WT, wild type

Abstract

The null mutation of cardiac Na⁺-Ca²⁺ exchanger (NCX1) gene in mice caused death of embryo *in utero* at embryonic day (ED) 9.0-9.5 and this embryonic lethality appears resulted from abnormal heart development. In the present study, we investigated whether transgenic re-expression of NCX1 in mutant cardiac myocytes could rescue these lethal defects. Transgenic mice expressing the canine NCX1 in a cardiac specific manner were bred into the NCX1 knock-out background but did not prevent the fetal lethality associated with the NCX1 null allele. However, the NCX1 knock-out embryos with an NCX1 transgene survived with heart beatings until ED 10.5 which was one day longer than the survival of the NCX1 knock-out embryos (ED 9.5). At ED 10.5, however, the partially rescued NCX1 embryos might have succumbed to the lack of an organized vasculature in the yolk sacs. The placental labyrinth layer was reduced in size and largely avascular. The transgenic re-expression of NCX1 rescued heart beatings and survived longer, but was still insufficient for the mice to be completely rescued. Importantly, NCX1 was observed to express in

the yolk sac and the placenta of wild type mice. The results suggest that defects in extra-embryonic compartments are causal to the lethality, and that NCX1 may play an important role in establishing vascularization in extra-embryonic tissues.

Keywords: antiporters; apoptosis; blood vessels; development, embryo and fetal; gene targeting

Introduction

Na⁺-Ca²⁺ exchanger 1 (NCX1) is known to play an essential role in Ca²⁺ homeostasis in cardiac muscle cells (Lee, 1985; reviewed in Blaustein and Lederer, 1999). In particular, NCX1 expression is highly restricted to the early embryonic heart and serves an important role in heart development (Koushik *et al.*, 1999; Linask *et al.*, 2001). Moreover, high levels of NCX1 expression and Na⁺/Ca²⁺ exchange activities have also been found in other tissues, such as brain and kidney (reviewed in Friedman, 1998; Sakaue *et al.*, 2000). Ca²⁺ homeostasis in these cells is essential to their function and there are various mechanisms to control intracellular calcium (Chin H, 1998). The functions of NCX1 in these cell types may be also important in the regulation of intracellular Ca²⁺. These facts suggest that the Na⁺-Ca²⁺ exchanger may have a universal function of acting as a major pathway for the extrusion of Ca²⁺ from the cell interior and protecting cells from Ca²⁺ overload (reviewed in Blaustein and Lederer, 1999).

The null mutation of the NCX1 gene caused lethality in mice embryos (Cho *et al.*, 2000; Wakimoto *et al.*, 2000; Koushik *et al.*, 2001; Rheuter *et al.*, 2002). Within embryonic day (ED) 9.0-9.5, the heart of the knock-out (KO) mice showed an irregular contraction and a pericardial effusion (Cho *et al.*, 2000). The NCX1 KO embryos also showed an apoptotic cell loss in the heart that could be caused by impaired regulation of intracellular calcium. These observations suggest that NCX1 is critical to early cardiac development. Other groups also reported similar cardiac defect induced lethality in the NCX1 KO mice (Wakimoto *et al.*, 2000; Koushik *et al.*, 2001). Recent report showed that transgenic re-expression of a NCX1 isoform was insufficient to rescue a lethal phenotype of NCX1 KO mice (Conway *et al.*, 2002). Taken together, these findings demonstrate that NCX1 plays a specific func-

tion in the heart during embryonic development.

Functional analysis of NCX1 ablation was limited, as the KO mice died at mid-gestation. Our study aimed to determine whether the re-expression of a NCX1 transgene in the embryonic heart could normalize the cardiac phenotype of the KO mice and rescue the KO mice from early embryonic lethality. We analyzed mice lacking endogenous NCX1 but expressed the NCX1 transgene in a cardiac specific manner.

Materials and Methods

Animals

The transgenic mice used in this study were developed by Philipson and colleagues (Adachi-Akahane *et al.*, 1997), and expressed the canine NCX1 gene under the control of the cardiac specific α -myosin heavy chain gene (α -MHC) promoter. Briefly, transgenic mice were obtained from pronuclear microinjection of the construct into C57BL/6xC3HF1 mouse egg. Mice heterozygous for the NCX1 gene (NCX1^{+/-}; C57BL/6x129F1), previously developed (Cho *et al.*, 2000), were intercrossed with the transgenic line to obtain mice that lack one copy of the NCX1 gene and contain one copy of the transgene (NCX1^{+/-;Tg/+}). Mating of those double heterozygous mice resulted in the offspring with different genotypes, *i.e.*, wild-type (WT), heterozygous, or homozygous for the NCX1 KO (NCX1^{+/-}, NCX1^{-/-}), and heterozygous or homozygous for the NCX1 transgene (NCX1^{Tg/+} or NCX1^{Tg/Tg}). To avoid the confusion of names, we will define the homozygous mice for NCX1 gene carrying the transgene as partially rescued mice and the homozygous mice for NCX1 gene without the transgene as KO mice. All mice were genotyped by PCR analysis of tail clip DNA. A total of 32 female NCX1^{+/-;Tg/+} mice and 17 female NCX1^{-/-} mice were used.

Heart beating

Heart rates were scored microscopically on embryos obtained from mating of double heterozygous mice (NCX1^{+/-;Tg/+}). Pregnant mice were killed by cervical dislocation. Embryos were harvested and placed in a chamber superfused with PBS at 37°C.

Reverse transcription (RT)-PCR analysis

Total RNA was isolated from ED 9.5 embryos. Using oligo-dT and AMV reverse transcriptase (Promega), first-strand cDNA was synthesized. Sense primer (bases 1742-1760) and antisense primer (bases 2171-2192) were used to generate PCR products. The amplification was performed using a thermal cycler Model

480 (Perkin Elmer) under the following conditions: initial denaturation at 94°C 1 min, followed by 38 cycles of 15 s at 94°C, 30 s at 58°C, and 30 s at 72°C. The PCR products were analyzed by electrophoresis on 1.5% agarose gels.

Histology and immunohistochemistry

Entire conceptus and placentas were fixed overnight in 4% paraformaldehyde in PBS. After rinsing in PBS and dehydration in an increasing ethanol series, embryos were embedded in paraffin according to standard procedures. Paraffin sections (6 μ m) were prepared and stained with hematoxylin and eosin (H&E) for the histological analysis. For whole mount staining with an anti-CD31 (PECAM) antibody (Pharmingen) was performed in WT and mutant conceptus at ED 9.5. Briefly, entire conceptus was fixed in 4% paraformaldehyde overnight at 4°C, blocked in PBT (5% goat serum and 0.2% Triton X-100 in PBS). After incubation with 10 μ g/ml of the anti-PECAM antibody in PBT at 4°C overnight, the conceptus was washed and incubated with a peroxidase-conjugated goat anti-rat antibody at 4°C overnight. After washing, the conceptus was stained with DAB (DAKO). For immunohistochemistry, sections were deparaffinized in xylene and rehydrated through alcohol series, and washed in PBS. The sections were blocked with 5% goat serum in PBS for 40 min, and then incubated with a rabbit polyclonal anti-NCX1 antibody, 1:100 (RDI) at 4°C overnight. After washing in PBS, the sections were incubated with a fluorescein-labeled anti-rabbit antibody (KPL) for 1 h at room temperature. The sections were washed in PBS, and mounted for viewing and photography with a fluorescence microscope.

Apoptotic cells

Apoptotic cells were identified by TUNEL assay on the sections of ED 9.5 conceptus and placenta using the Apoptag in situ apoptosis detection kit (Intergen) as described by the manufacturer. The number of apoptotic cells was scored using H&E-stained sections microscopically under 400 $\times$ magnification and scored the number of apoptotic cells per 100 nuclei. The apoptosis was also confirmed by TUNEL method.

Statistics

All quantitative data are expressed as mean $\pm$ SE.

Results

Generation of rescued mice

To determine the effect of cardiac-specific rescue on

the cardiovascular defect in the NCX1 KO mice, the transgenic lines expressing NCX1 in the heart were crossed with heterozygous NCX1 KO mice. Mice lacking both copies of the NCX1 gene and NCX1 transgenic lines expressing the canine NCX1 under the control of the α -MHC promoter were previously

generated (Adachi-Akahane *et al.*, 1997; Cho *et al.*, 2000). The protocol assumed that using the NCX1 gene from a different species would resume the function in mice as the homologs are highly conserved across species and also known to be quite similar in physiological function (Maxwell *et al.*, 1999). The he-

Table 1. Offspring obtained from rescue experiment with transgenic NCX1 (NCX1^{+/+}Tg⁺ × NCX1^{+/-}Tg⁺).

NCX1 gene	NCX1 ^{+/+}		NCX1 ^{+/-}		NCX1 ^{-/-}	
	+	-	+	-	+	-
NCX1 transgene	+	-	+	-	+	-
ED 9.5	4	5	9	7	3*	4*
ED 10.5	11	8	12	9	6*	2
ED 11.5	3	2	4	5	5	-

*With similarly abnormal morphology, * Severely disfigured and mostly absorbed.

Figure 1. RT-PCR detection of mRNA expression of NCX1 in the heart of ED 9.5 embryos. Note the increased levels of NCX1 expression in the partially rescued heart. The size of diagnostic fragments for mRNA is indicated. Samples in lanes RT+ contained reverse transcriptase while RT- did not. M, molecular weight marker.

Figure 2. Morphological and histological analysis of embryo. A-D. Morphology of WT (NCX1^{+/+}) and partially rescued embryos (NCX1^{-/-}Tg^{+/+}). At ED 9.0, both embryos were similar in developmental stage (A). However, the partially rescued embryos showed growth retardation at ED 9.5 (B), ED 10.0 (C), and ED 10.5 (D). Note the pericardial dilation of the partially rescued mice. E-G. Determination of apoptosis in WT (E), partially rescued (F), and KO (G) heart at ED 9.0. Sections through the ED 9.0 heart, showing the normal development of myocardium and endocardium in WT and partially rescued ventricles while apoptotic cells were found on the myocardium of KO ventricle. Arrow indicate apoptotic positive cell. V, ventricle.

terozygous mice for the NCX1 gene were first mated with the NCX1 transgenic lines. Then the genotypes of NCX1^{+/-;Tg/+} were intercrossed to generate mice that expressed a transgenic NCX1 and were homozygous for the NCX1 KO (genotypes: NCX1^{-/-;Tg/+} or NCX1^{-/-;Tg/Tg}). Genotyping by PCR analysis of embryos on ED 9.5 verified the presence of embryos with different genotypes at the expected Mendelian ratio (Table 1). However, all NCX1^{-/-} animals with or without the transgenic NCX1 died *in utero*, indicating that the transgenic expression of the NCX1 failed to rescue the lethal embryonic condition of the NCX1 KO animals. The important finding, however, was that the NCX1 embryos that carried transgenic alleles survived until ED 10.5 and were lost at ED 11.5 (Table 1). This is one day-extension of the survival of NCX1 KO embryos by the transgenic expression of NCX1 in the heart, compared to the survival of NCX1 KO embryos without the transgenic NCX1. To ensure that the transgenic NCX1 was expressed in the embryonic heart, a RT-PCR was performed. While no expression was observed in the KO heart, the partially rescued heart expressed NCX1 (Figure 1).

Gross and histological phenotypes of the partially rescued mice

The transgenic rescue of the cardiovascular defect

permitted the NCX1 KO embryos to survive one day longer than the KO embryos without the transgenic NCX1 (Figure 2A-D). The partially rescued embryos displayed growth retardation and pericardial swelling similar to that of the NCX1 KO embryos at ED 9.5. In addition, the partially rescued embryos recovered at ED 9.5-10.5 were the same size as the WT embryos at ED 9.0, indicating that the growth retardation began after ED 9.0. However, the increased number of somites and the presence of a forelimb bud at ED 10.5 indicated that the partially rescued embryos were further developed, a phenomenon that had never been observed in the NCX1 KO embryos. By ED 11.5, the deformation rapidly aggravated and all partially rescued embryos were almost resorbed (data not shown). The most prominent phenotype of the partially rescued embryos was the extension of the heartbeat. The partially rescued mice were observed to have a regular heartbeat until ED10.5, while the heartbeat of the KO mice was irregular at ED 9.0 and stopped at ED 9.5 (Figure 3). The heart rates of the WT, the partially rescued, and the KO mice were 72±6 (*n* = 8), 46±5 (*n* = 6), and 4±1 (*n* = 4) beats per minute (bpm) at ED 9.0, and 76±6 (*n* = 8), 57±5 (*n* = 4), and 0 bpm (*n* = 4) at ED 10.5, respectively. Although the partially rescued mice showed rhythmic contractions in the heart, heart rates of the

Figure 3. Morphology of WT, partially rescued, and KO conceptus at ED 9.5 (A-C) and ED 10.5 (D-F). Note that vitelline vessels were visible in WT embryo (arrows; A, D) but missing in partially rescued (B, E) and KO (C, F) yolk sacs. There were also excessive folds on the surface of the mutant yolk sacs. p, placenta; y, yolk sac.

partially rescued mice were slightly reduced. These results implied that the cardiac NCX1 was a requirement for the generation of heart beating during early cardiac development and there might be some other reasons for the lethality of the KO and the partially rescued mice.

Histological analysis of ED 9.0 embryos indicated that the heart defect seen in the KO embryos was corrected in the partially rescued embryos. While the morphology of the partially rescued myocardium appeared similar to that of the KO embryos, the apoptotic cells observed in the heart of the KO embryos were not detected in the partially rescued embryo (Figure 2E-G). The percent of apoptotic cells in ED 9.0 heart were $2.8 \pm 0.6\%$ in WT embryos ($n = 4$), $3.0 \pm 0.7\%$ in the partially rescued embryos ($n = 5$), and $9.8 \pm 0.9\%$ in the KO embryos ($n = 4$), respectively. Taken together, these results indicated that the transgenic NCX1 improved the heart defects observed in the NCX1 KO embryos, and that the lethality of the KO and the partially rescued mice was unlikely to be caused by defects in heart function.

Defects in yolk sacs

The partially rescued embryos were not only severely disfigured, but their yolk sacs were also wrinkled, shrunken, anemic, and lacked vasculature at ED 9.5-10.5 (Figure 3). Formation of the yolk sac blood vessels begins with the fusion of blood islands into a blood vascular plexus. This network of thin tubules

then undergoes morphogenetic events such as remodeling to generate larger, branched vessels [16] (reviewed in Patan, 2000; Risau, 1997). While the large vessels were found in the WT yolk sac (Figure 3A), no definitive vasculature was formed in the KO and the partially rescued yolk sac at ED 9.5 (Figure 3B-C). This phenotype became more definitive at ED 10.5 (Figure 3D-F). The pale appearance and the abnormal vasculature in the KO yolk sac were not corrected by the transgenic NCX1. Whole-mount PECAM staining of WT yolk sacs at ED 9.5, which is specific marker for the vascular endothelial cells, confirmed the formation of the large vitelline and the fine network of small vessels (Figure 4A). In contrast, well-defined large vessels could not be seen in the KO and the partially rescued yolk sac but some primitive vascular cells were stained positively (Figure 4B-C).

Histological analysis showed that in the mutant embryos with and without the transgenic NCX1, the mesoderm and endoderm of the yolk sac were separated with few observed areas of contact between the two layers, unlike the WT yolk sac (Figure 4D-F). Moreover, some endothelial cells were observed in the mutant yolk sac, but these cells did not form the vascular channels. Although the blood vessels were defective in the mutant yolk sac, they contained fetal blood cells between the two layers, indicating that hematopoiesis was normal but blood supply to the embryo was impaired (Figure 4E-F).

To demonstrate that the lack of vascularization was

Figure 4. Histological analysis of yolk sac. A-C, whole mount anti-PECAM antibody staining of WT (A), partially rescued (B), and KO (C) yolk sac. Note the lack of branching vitelline vessels in partially rescued and KO yolk sac. D-F, histological analysis of yolk sac at ED 9.5. Transverse sections of WT (D), partially rescued (E), and KO (F) embryos revealed the abnormal yolk sac morphology, indicating the yolk sac phenotype may be the cause of lethality. En, extra-embryonic endoderm; Me, extra-embryonic mesoderm; Am, amnion. Scale bars: 100 μ m.

due to the defect of differentiation in vascular precursor cells, the expression of NCX1 in the yolk sac was examined. Previous studies showed that NCX1 gene consisted of a cluster of six exons (designated as A, B, C, D, E, and F), generating various isoforms in the large intracellular loop of the protein and (Kofuji *et al.*, 1994). RT-PCR was performed using total RNA prepared from the WT embryo and the WT yolk sac at ED 9.5 and sense primer (bases 1742-1760) and antisense primer (bases 2171-2192) were designed to detect the variable region covering the large intracellular loop of NCX1 gene. Agarose gel electrophoresis of PCR products showed that NCX1 was not

only expressed in the heart, but that isoforms of the NCX1 were also detected in the yolk sac and the whole body (Figure 6). The PCR product from the yolk sac was shorter than those in the heart and, when sequenced, it was recognized as alternative spliced variant of NCX1 gene (data not shown). The transcript in the yolk sac consisted of exons B, D, and F in the large intracellular loop of NCX1 gene, which is identical to NCX1.7 according to the terminology proposed by Quednau *et al.* (data not shown). Only a single isoforms of NCX1 was found in two different mRNA preparations. This result does not, however, eliminate the possibility that there existed other isoforms of the NCX1.

Figure 5. Expression of NCX1 in ED 9.5 embryos. Total RNA harvested from WT and KO was used to RT-PCR analysis. Note the spliced-variant in the yolk sac. The size of diagnostic fragments for NCX1 mRNA is indicated. B, whole embryo except heart; H, heart; Y, yolk sac; M, molecular weight marker.

Defects in placentas

At ED 9.0-9.5, the labyrinth layer of the placenta develops, wherein extensive intermingling occurs between maternal and fetal blood vessels (reviewed in Cross *et al.*, 1994). The first step in the development of the placenta is the fusion of chorion and allantois. Thereafter, allantoic vessels invade into the chorionic plate, which is then converted into the labyrinth layer. Histological analysis of the placenta from ED 9.5 embryos showed that the chorioallantoic fusion was not affected in the mutant embryos while the essential labyrinth layer was not formed in the KO and the

Figure 6. Histological analysis of placenta. A-C, histology of placenta from WT (A), partially rescued (B), and KO (C) embryos at ED 9.5. Note that the extensive intermingling of maternal (containing small enucleated erythrocytes: red arrows) and embryonic blood vessels (large nucleated erythrocytes inside: green arrows) occurs in the labyrinth layer of WT placenta (A) while blood vessels of KO and partially rescued placenta did not penetrate into the labyrinth layer (B, C). D-F, TUNEL assay on placenta of WT (D), partially rescued (E), and KO (F) embryos at ED 9.0 showed the high degree of apoptosis, as indicated by brownish nuclei stains. Positive stains of fragmented nuclei were found on the allantoic vessels of placenta, suggesting that the reduced labyrinth layer were due to the loss of invasive cells. la, labyrinth layer; ch, chorion. Scale bars: 100 μ m.

Figure 7. Expression of NCX1 in placental vasculature. A-D, immunofluorescence of transverse sections of WT and partially rescued placentas stained with anti-NCX1 antibody. At ED 9.5, NCX1 was normally found on the wall of fetal blood vessels in WT placenta (A,C: arrows) but not in partially rescued placenta (B,D). C and D are amplified view of A and B, respectively. Positive stains in the B and D showed the presence of maternal blood vessels. la, labyrinth layer; ch, chorion. Scale bars: 100 μ m.

partially rescued embryos (Figure 6A-C). The WT placenta, on the other hand, showed a normal capillary formation while the KO and the partially rescued placenta exhibited a compact nonvascularized cell layer. TUNEL staining also showed that some chorio-allantoic cells in the KO and the partially rescued placentas were apoptotic at ED 9.0 (Figure 6D-F). These anomalies may affect the exchange of gases and nutrients between the fetus and the mother, thereby contributing to mid-gestational death.

To verify the expression of NCX1 in vascular endothelial cells, immunohistochemistry was performed on ED 9.5 embryos (Figure 7). In the WT embryos, the NCX1 was observed in the endothelial cells of the fetal blood vessels and some chorionic cells also stained positively (Figure 7A and C). In contrast, mutant embryos exhibited no NCX1 was not observed in the partially rescued placenta (Figure 7B and D). The positive stains shown in the partially rescued placenta was likely due to the maternal vessels. These results indicate that NCX1 might play an important role in the extra-embryonic vasculogenesis.

Discussion

NCX1 plays an essential role in the regulation of

intracellular Ca^{2+} and cardiac contractility (Cho *et al.*, 2000). Ablation of NCX1 leads to defects in cardiac development. Although this finding is suggestive of importance of NCX1 in cardiac function, several issues remain to be clarified. Importantly, NCX1 is most highly expressed during late developmental stages, implying the critical role of NCX1 in various organs (Kouban *et al.*, 1998; Qu *et al.*, 2000). Also, another group reported that NCX1 deletion had nothing to do with the heart development but related to heart beating and that the cause of embryonic lethality was due to the lack of a circulation (Koushik *et al.*, 2001). Although this difference might arise from the different targeting strategy and/or different genetic backgrounds, it is possible that there might be other unknown factors to comprise embryo development. The present study thus aimed to rescue the NCX1 embryo from the lethality by cardiac specific expression of transgenic NCX1 and to examine NCX1 function during late developmental stages. If the lethal phenotype of NCX1 KO mice was only due to insufficient cardiac function and/or defects in cardiac development, the re-expression of NCX1 in heart should rescue the lethality.

There was a distinct difference in cardiac function between the KO and the partially rescued mice. The heartbeat of the KO mice was observed to be ir-

regular at ED 9.0, eventually stopping at ED 9.5. In contrast, the partially rescued embryos maintained a regular heartbeat at ED 9.5 and survived up to ED 10.5. In addition, no sign of apoptotic cell death was found on heart of the partially rescued mice, indicating that apoptosis shown in the KO heart was likely due to the deletion of NCX1 in heart and NCX1 transgene seems sufficient for cardiac development. Despite the rhythmic contraction, the heart rates of the partially rescued embryo were slightly reduced, compared with those of the WT, implying that another factors might compromise normal cardiac function. Although the cardiac rescue did not change the lethality of NCX1 KO mice, the partially rescued mice gave some clues on the cause of the lethality of the NCX1 KO mice.

In many genetically modified mutant mice, severe developmental defects and growth retardation are frequently associated with extra-embryonic defects leading to lethality (Yang *et al.*, 1995; Schorpp-Kistner *et al.*, 1999; Schreiber *et al.*, 2000). During early embryogenesis, the survival of an embryo is dependent on the formation and maintenance of a functional placenta and yolk sac. In mice, the definitive placenta is formed where the embryonic tissues actually invade beneath the maternal epithelial cell layer and into the stromal tissue to make direct contact with the maternal capillary bed (reviewed in Rinkenberger *et al.*, 1997). The labyrinth layer is formed by the fusion of allantois and chorion, which originate from the extra-embryonic mesoderm and extra-embryonic ectoderm. The fusion of allantois and chorion is an important prerequisite for the formation of the labyrinth layer where nutrient and gas exchanges between the fetal blood vessels and the maternal blood sinuses take place (reviewed in Patan *et al.*, 2000). The histology of mutant placentas demonstrates that the absence of NCX1 affected the morphogenesis of the placental labyrinth (Figure 6). Mutant embryos that were able to develop until the stage when the chorioallantoic placenta became responsible for embryo nutrition died from failure to establish the functional placenta. The defect in the labyrinth layer was not due to the loss of either chorion or allantois since they were not only present but also normally fused. After their fusion, the allantoic cells invade the placenta, forming the fetal blood vessels. However, in the mutant placentas, this invasion was drastically reduced and eventually failed to differentiate into the complex vascular network of the labyrinth layer. In accord with the decreased invasion in the labyrinth, a TUNEL assay showed the degeneration of mesenchymal cells in the ED 9.0 mutant placenta, when the morphological defect was not apparent. The described placental abnormalities of the KO and the partially rescued embryos, therefore, may contribute to the lethality.

The placental defects alone could not explain the severe retardation of the mutant embryo at ED 9.5, because some mutant mice showed similar placental defects with only mild growth retardation (Guillemot *et al.*, 1994; Ma *et al.*, 1997). Another mutants even showed only slight growth retardation in which the chorioallantoic fusion was impaired (Chang *et al.*, 1999; Hunter *et al.*, 1999). On the other hand, defects in the yolk sac frequently caused fetal growth retardation (Healy *et al.*, 1995). The yolk sac is the first site of hematopoiesis and is the major source of blood cells. Formation of the yolk sac blood islands and their subsequent remodeling into a functional circulatory system become critical for the fetal survival beyond ED 9.5. Indeed, a defect in the yolk sac of the KO and the partially rescued mice could be demonstrated. The primary capillary plexus was normally formed before ED 9.0 and contained blood cells in both the WT and the mutant yolk sac. Thereafter, however, the mutant yolk sac did not form a large vascular structure, indicating that further vasculogenesis and angiogenesis did not occur in the mutant yolk sac. Thus, the death of the mutant embryos could be due to the failure to initiate a functional blood circulation in the yolk sac that lacked an organized vascular network and subsequent osmotic imbalance could explain the cardiac dilation, which was the phenotype of the mutant heart.

Recently, four NCX1 KO mice were reported that NCX1 is important to embryo development. These mice showed similar but somewhat different phenotypes. One of these mice showed that NCX1 is important to heart beating and proposed that NCX1 was required for formation of beating heart and that simple diffusion of maternal oxygen and nutrient was sufficient for survival to ED 11.5 in their KO mice (Koushik *et al.*, 2001). However, other three mice showed that NCX1 was more critical to heart development because the heart of KO mice displayed spontaneous beating at ED 9.0-9.5. Similar strategy of transgenic re-expression of NCX1 in NCX1 KO mice was recently reported that failed to rescue the KO mice (Conway *et al.*, 2002). No coexpression of two different NCX1 isoforms (NCX1-U and NCX1-AF) in the heart that were normally expressed in embryonic heart was suggested as the cause for the failure of transgenic rescue of NCX1 KO mice. From this point of view, our use of NCX1-U as a transgene is the reason for failure to rescue the NCX1 KO phenotype. However, there wasn't any consideration for the expression of NCX1 spliced variant in the yolk sac. Secondly, there is major difference between two sets of NCX1 KO mice experiments. Our transgene NCX1 KO mice clearly showed partial rescue from ED 8.5 to ED 9.0, even ED 10.0 in comparison with the treated mice with no rescue (Koushik *et al.*, 2001).

This difference could tell us exact delaying of survivals in partially rescued mice. Although the reason for the differences is not clear, we believe that it was due to the different mice strains, because similar targeting strategy leads to different phenotypes (Koushik *et al.*, 2001; Rheuter *et al.*, 2002). Moreover, the rescue of apoptosis in the partial rescued heart implies that the apoptosis in the KO heart was not related to the gene targeting strategy (for example, effects of neomycin cassette).

The defect in the yolk sac circulation may have been due to the insufficient heart function of partially rescued mice that were not strong enough to deliver blood to periphery. It is likely that other factors contributed for vascular defects in the extraembryonic compartment. First, defects in vascular formation in the yolk sac were not described for mutant embryo for Hand1 (Riley *et al.*, 1998), although heart formation arrested at the similar stage, and even some mutant mice showed extraembryonic defects in spite of their beating heart (Wang *et al.*, 1997; Shreiber *et al.*, 2000; Ishikawa *et al.*, 2001). Second, the extraembryonic mesoderm, generated during gastrulation, gives rise to blood islands and blood vessels associated with the visceral yolk sac (Patan, 2000). This mesoderm also produces the allantois, the precursor of the umbilical blood vessels, suggesting that the formation of vasculature in the extraembryonic compartment occur the placenta. However, vascular defects are observed in the placenta at ED 9.0, which is prior to obvious cardiac defects. Third, in accordance with this result, NCX1 expression was detected in the WT yolk sac and whole embryo. Although NCX1 transcript in the yolk sac was relatively small amount, it was unexpected because Koushik *et al.* reported that NCX1 was expressed in a cardiac-specific pattern within the early mouse embryo until ED 11.0 (Koushik *et al.*, 1999). Wakimoto *et al.* (2001) also reported that alternatively spliced isoforms of NCX1 were expressed in the whole embryo, as well as in the heart of ED 10.5 mice. Although these time differences were presumably due to the mice strain, we believe that it might result from sensitivity of used technique detecting the low abundant transcripts. For example, Koushik *et al.* showed cardiac restricted NCX1 expression at ED 11.0 using in situ hybridization (Koushik *et al.*, 2001). However, they detected NCX1 in whole embryo at ED 11.0 by PCR analysis (Koushik *et al.*, 1999). Detection by in situ hybridization likely requires that the level of target mRNA within the cell surpasses a concentration threshold (Schneider *et al.*, 1998). Indeed, we could not detect the transcript in yolk sac and whole embryo with RT-PCR at 25 cycles (data not shown).

A question arises about the function of the NCX1 in vascular development. Although several reports

have indicated the contribution of $\text{Na}^+\text{-Ca}^{2+}$ exchange to Ca^{2+} regulation in endothelial cells and smooth muscle cells (Smith *et al.*, 1994; Teubl *et al.*, 1999), its role in the functional vasculature differentiation is unknown. Moreover, the expression of NCX1 in the fetal blood vessels and the yolk sac is not indicative of its role in the differentiation into fetal vessels. In addition, while apoptotic mesenchymal cells were found in the placenta of the mutant mice, whether some of these apoptotic mesenchymal cells are progenitors of the endothelial cell is unknown. It is tempting, however, to speculate that the failure to differentiate into the definitive placenta may be related to NCX1 function in a critical cell population (such as preventing apoptosis in these cells) and that loss of NCX1 function in a yet unknown manner disrupts the maintenance of the cellular stability of these cells. Likewise, the failure of the yolk sac vascularization may be caused by the same molecular mechanism.

In summary, our study suggests that (i) the cardiac-specific expression of the NCX1 gene could rescue the heart phenotype of the NCX1 KO embryo, and that (ii) in contrast to the prolonged heart function of the partially rescued embryos, the presence of transgenic NCX1 did not change the early lethality of the KO embryos, and that (iii) the lethality of KO and partially rescued mice was caused by the extraembryonic defects. These findings suggest that NCX1 may be critical to the implantation process including the integrity of blood vessels in the yolk sac and the differentiation of the functional placenta.

Acknowledgement

We thank Gou Young Koh for extensive discussion and Myoung Phil Kong for animal care. This work was supported by Korea Science and Engineering Foundation (KOSEF 00040102), Korea Research Foundation Grant (KRF-2000-015-DS0035), and National Creative Research Initiative Program of Korea.

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