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ORIGINAL ARTICLE

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Loss of function of the *Prx1* and *Prx2* homeobox genes alters architecture of the great elastic arteries and ductus arteriosus

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Abstract *Prx1 (MHox)* and *Prx2 (S8)* are non-clustered homeobox genes that are expressed in a complex, mostly mesenchyme-specific pattern throughout embryogenesis. The expression pattern and gene-targeted mice previously revealed a major role for *Prx1* in skeletogenesis. In addition, specific and high expression of both *Prx* genes was reported in the developing cardiovascular system, predominantly in prospective connective tissues of the heart and in the great arteries and veins. We examined embryos of previously generated gene-targeted mice. *Prx2-/-* mutants were viable and did not show cardiovascular malformations. Intracardiac morphology of *Prxl-/-* and *Prx1/Prx2*-combined null mutants also appeared normal throughout development. However, the *Prx1-/-* and *Prx1/Prx2* double-null mutants showed a vascular abnormality with an abnormal positioning and awkward curvature of the aortic arch in addition to a misdirected and elongated ductus arteriosus, and in two of seven combined mutants, an anomalous retro-oesophageal right subclavian artery. Generally, all great arteries appeared to run somewhat tortuously through the surrounding mesenchyme. The vascular histology and vessel wall thickness were normal in all mutants. *Prx1-/- and Prx* double-gene-targeted mice revealed similar spectra of vascular anomalies, but double mutants appeared to be more seriously affected. The current findings suggest that other genes may compensate for the loss of *Prx* in the heart, but, in contrast, our data support a role for *Prx* in the development of vascular and perivascular matrix.

Key words Paired-related homeobox · Heart development · Pharyngeal arch arteries · Vascular matrix

Abbreviations *Ao* aortic arch, *AsAo* ascending aorta, *BA* brachiocephalic artery, *CA* carotid artery, *DA* ductus arteriosus, *DsAo* descending aorta, *LSA* left subclavian artery, *LV* left ventricle, *MV* mitral valve, *OE* oesophagus, *PA* pulmonary artery, *PT* pulmonary trunk, *RRSA* retro-oesophageal right subclavian artery, *RSA* right subclavian artery, *RV* right ventricle, *T* trachea, *TV* tricuspid valve

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Introduction

The paired-related homeobox genes *Prx1* and *Prx2* encode proteins containing a homeodomain that is highly homologous to that of the *Drosophila* segmentation gene *paired.* Since these genes were cloned by different strategies, they have been given numerous names. Names currently used for homologues include *MHox, Phox 1, Pmx, rhox,* and *K2*; all are here referred to as *Prx1*. The former *S8* gene is now called *Prx2* (see [15] and references therein).

Studies on the embryonic expression patterns of *Prx1* and *Prx2* revealed that they were expressed, from very early stages onwards, in a wide variety of tissues [8, 13, 15, 20, 21]. Functions of the genes are probably diverse, depending on timing and type of the cells in which they are expressed. Analysis of *Prx1* interactions at the molecular level with specific *cis* sequences and protein cofactors has strengthened this concept.

Prx1 was shown to enhance expression of genes containing CArG sequences (also known as serum response

elements) in their promoters [9, 18]. The CArG motif is found in serum-inducible genes such as *c-fos* [29], *Krox20/EGR2* [23], interleukin-2 receptor [24] as well as in many muscle-specific genes [18 (see also references therein), 20], including α-actins ([4, 19] and SM22α [12]. The PRX*1* homeodomain alone was sufficient for enhanced expression of the aforementioned genes [9]. Up-regulation was thought to be accomplished by PRX-mediated enhanced binding of serum response factor (SRF), and other MADS-domain proteins to the CArG elements [9], but this mechanism has recently been disputed [18]. *Prx1* has also been reported to be a repressor. In vitro co-transfection studies with the rat *Prx1* homologue demonstrated that it represses promoter activity of collagen Iαl and osteocalcin genes [11]. This suggests that *Prx1* is important for extracellular matrix modulation.

Although initial molecular research focused on putative roles for *Prx1* in muscle differentiation and regulation of contractile machinery, more recent studies on embryonic expression patterns revealed that the role of *Prx* genes in myocyte differentiation is probably limited. *Prx* genes are highly expressed in mesenchymal tissues throughout development (pharyngeal arches, limb buds, cranial mesenchyme, dermis, and bone-forming tissue). These patterns suggested a major role in connective tissue development, and myocytes generally showed no, or very low, transcript levels [13, 15, 20, 21]. Recent studies by our group examined the expression of *PRX1* and *PRX2* in the chick embryonic vascular system. Our results did not support major role for *PRX* genes in regulation of smooth muscle contractile proteins, but suggested a role in matrix modulation instead [2]. Therefore, the significance of *Prx* genes in skeletal, cardiac, and smooth muscle differentiation is debatable.

The developing cardiovascular system was one of the organ systems in which high and specific transcript levels of *Prx* genes were reported. Both *Prx1* and *Prx2* were expressed in similar patterns at early stages in the developing vascular system, but patterns clearly diverged later. *Prx1* was highly expressed in the developing atrioventricular and outflow tract cushions and valves, epicardium, adventitia of veins and muscular arteries, as well as in the adventitia and media of the great elastic arteries. *Prx2* showed only moderate levels in arterial adventitial cell layers, but its expression was remarkably high in the ductus arteriosus (DA), clearly delineating this muscular vessel from the adjoining elastic great arteries [2, 15].

Homeodomain transcription factors, like *Prx* genes, are commonly recognised as important regulators of embryonic development, and functional deletions of many homeobox genes are incompatible with normal development and life. In an attempt to further elucidate *Prx* function, gene-targeted mice were recently generated for both loci. *Prx1-/-* mice mainly exhibit malformations in craniofacial, vertebral and limb skeletal structures; newborns have respiratory distress and die soon after birth [5, 17]. *Prx2-/-* mice are normally viable, without any obvious morphological abnormalities. Yet, physiological studies on isolated *Prx2* mutant hearts demonstrated altered cardiac function (M.J. Kern, pers. commun., 1997). *Prx1/Prx2* double-mutant mice show an aggravated *Prx1* phenotype and, in addition, novel abnormalities in craniofacial and limb skeletogenesis [16, 27]. Like the *Prx1-/-* mice, the double mutant *(Prx1-/-, Prx2-/-)* mice died within 24 h after birth.

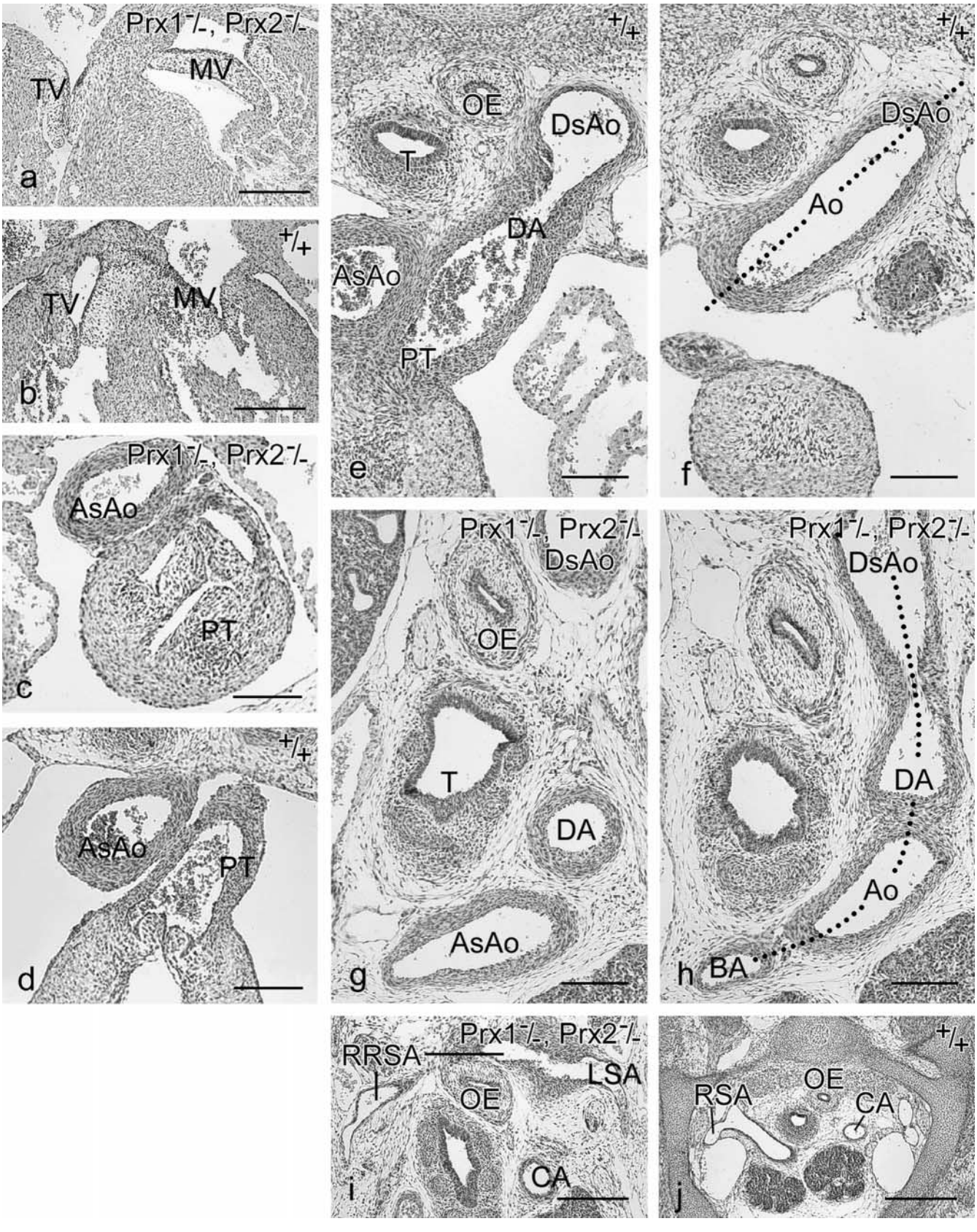
Because *Prx* genes are highly expressed throughout cardiovascular development, and in very specific patterns, we decided to scrutinise the single *Prx*-gene-targeted mice as well as *Prx1/Prx2*-combined null mutants for cardiovascular anomalies. Results of the present study show that despite high cardiovascular expression levels of these putative important regulatory genes during normal development, loss of function did not generate intracardiac abnormalities. However, *Prx1-/-* and *Prx1-/-, Prx2-/-* mice revealed a spectrum of disorganised, oddly directed, and somewhat elongated great arteries. *Prx2-/-* mice showed an entirely normal cardiovascular morphology.

Materials and methods

The generation and genotyping of the single-gene-targeted mice for *Prx1 (Mhox)* [17] and *Prx2 (S8)* (Kern et al., submitted) are described elsewhere. *Prx1/Prx2*-combined null mutants were offspring of double heterozygous matings of the aforementioned strains [17]. Genotyping of offspring was performed as described by Lu et al. [16]. Animals evaluated in the present study were derived from multiple litters of multiple founders. Gene-targeted mice were compared with wild-type embryos of similar and different genetic backgrounds. All mice were kept and treated according to the Principles of Laboratory Animal Care (NIH publication #85-23, revised 1985).

Animals aged from E14.5 to newborns were killed and fixed in 4% paraformaldehyde, dehydrated and embedded in paraffin. Sections of 5–10 µm were cut and mounted serially onto glass slides. Great care was taken to standardise the angle of transverse sectioning with respect to the thoracic spine among both wild-type and mutant embryos. Morphological evaluations were based on standard haematoxylin/eosin-stained sections. A resorcin/fuchsin staining for elastic fibres [25] was applied to enable better delineation of the wall of the muscular DA amongst the adjoining elastic vessels, before graphic reconstruction of the arterial tree from the sections [28].

Fig. 1a–j Cardiovascular morphology of *Prx1-/-, Prx2-/-* embryos in comparison with wild type at E14.5. **a.** Despite previously described high expression levels of *Prx* transcription factors in the developing atrioventricular valves, double-gene-targeted embryos showed normal tricuspid and mitral valves at E14.5. *Bar* 250 µm **b.** Wild type appearance of tricuspid and mitral valves at E 14.5. **c, d.** Morphologically normal semilunar valves of the pulmonary trunk of a double null embryo (**c**) as compared with the wild type (**d**). *Bars* 200 µm **e, f** Standard transverse sections through the arterial pole of a wild type at the level of the ductus arteriosus (DA) (**e**) and the aortic arch (**f**). Note that the DA in the wild type is cut longitudinally and the aortic arch runs in a straight diagonal course (*dashed line*) through the thorax. *Bars* 150 µm **g, h** Comparison of transverse sections in a double mutant embryo, at similar levels to those in **c** and **d**, respectively. Note the almost transversely sectioned DA (**g**), the awkward sideways curvature of the aortic arch, and the altered disposition of the arteries in relation to each other (**h**). *Bars* 150 µm **i, j** Micrographs of an anomalous retro-oesophageal right subclavian artery (**i**), which branches off the aortic arch together with the left subclavian artery as compared with its normal origin from the aortic arch in the wild type (**j**). Bars **i** 250 µm, **j** 400 µm


Prx1-/-, Prx2-/-
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Prx1-/-, Prx2-/-
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Results

Prx1/Prx2 combined homozygous null mutants

Developmental stage E14.5

Hearts of *Prx1/Prx2* double-mutant mice did not reveal morphological anomalies in the youngest stages studied (E14.5, *n*=3). Myocardial organisation of atria and ventricles appeared completely normal in size, shape of trabeculae and free walls, and in ventricular septation. Owing to the *Prx* expression pattern a particular focus was on the examination of developing atrioventricular and semilunar valves (Fig. 1a, c), but no morphological differences in size, shape or gross cellular composition could be detected between the double-mutant and the wild-type hearts (Fig. 1b, d).

In contrast to the normal morphology of the heart, sections through the great arteries revealed anomalies in vascular orientation and direction within the thorax. In transverse sections of normal wild-type embryos, the pulmonary trunk and DA are characteristically sectioned longitudinally, showing the connection of the lumen through the pulmonary trunk, prospective DA, all the way through to the descending aorta (Fig. 1e). Transverse sections through the aortic arch of wild types reveal a straight diagonal course of the arch from its right and ventral origin towards its descending part at the left and dorsal sides with respect to the heart (Fig. 1f). However, in the most badly malformed double-gene-targeted mice, the pulmonary trunk was positioned slightly more vertically than in wild types, but this was much more the case for the DA, which ran from the pulmonary trunk in a cranio-dorsal direction towards the descending aorta. As a consequence, transverse thoracic sections showed more or less transverse/oblique sections through the DA, instead of longitudinal views (Fig. 1g). In addition to the abnormal course of the DA, the aortic arch was seen to run first laterally from right to left, before bending rather sharply in a dorsal direction, finally taking its usual caudal course as the descending aorta in the left side of the mediastinum (Fig. 1h). In mutants, the arteries branching off the aortic arch appeared to be set slightly further apart than in normal embryos, demonstrating an apparent elongation of the aortic arch itself. In one out of three double-mutant embryos studied at this stage, the abnormally coursing aorta and DA were accompanied by the presence of an anomalous retro-oesophageal right subclavian artery (arteria lusoria) (Fig. 1i, j).

Peripheral circulation (small arterioles and venules in the skin and other organs and structure) appeared completely normal. No anomalies were observed in the major veins running to the heart. We never observed rupture of blood vessels.

E16.5–E18.5

At later stages in development, the characteristic view of the great arteries in standard transverse thoracic sections was maintained in all wild-type animals studied. As in E14.5 embryos, the DA in E18.5 wild-type embryos was sectioned through its entire length (Fig. 2a) and the aortic arch ran in a straight diagonal course through the plane of section (Fig. 2b).

The great arteries in double-gene-targeted mice, however, retained the abnormal position and directions described for the E14.5 embryos. In addition to gross directional change of the DA and the awkwardly laterally curved aortic arch (Fig. 2c, d), the arteries took a slightly more sinuous course through the mesenchyme. Cross sections through the arteries sometimes showed irregular vessel walls instead of rather smooth ones, yet overall vessel wall thickness resembled that of wild-type mice, as was the case for the relative proportion of medial and adventitial cell layers. One of the animals studied for ages E16.5–El8.5 (*n*=4) had a retro-oesophageal right subclavian artery. Graphic reconstruction of sectioned embryos showed the sinuous great arteries and suggested that the DA and the aortic arch were of a greater length than in their wild-type counterparts (Fig. 2f, g). No abnormalities were seen in the major veins, nor were they obvious in smaller vessels in the periphery. Rupture of blood vessels was never noticed.

As had been the case at E14.5, embryos at later stages revealed a similar vascular phenotype to a lesser or greater degree, resulting in a spectrum of vascular malformations that can be attributed to the *Prx1-/-*, *Prx2-/-* genotype.

Intracardiac morphology in E18.5- *Prx1/Prx2* combined null mice was not different from that in normal embryos. Development of the atrioventricular and semilunar valves had progressed normally (Fig. 2e). No obvious anomalies in the patterns or structure of coronary vasculature were seen.

Prx1-homozygous null mice

Prx1 homozygous null mice (*n*=6) showed a phenotype very similar to the vascular phenotype described for *Prx1/Prx2* combined mutants (Fig. 3a, b). The spectrum of *Prx1*-related vascular anomalies, however, generally appeared less severe than in double mutants. We did not observe a retro-oesophageal subclavian artery in this group, and 2 of 6 embryos were indistinguishable from wild-type littermates. *Prx1* single mutants only live a few hours after birth.

Prx2-homozygous null mice

Prx2-homozygous mutants (*n*=7) of all stages studied revealed no cardiac anomalies when compared with wild-type controls. The heart was morphologically normal, showing no defects in its myocardial organisation, septa, coronary vasculature, or valves. Similarly, the great vessels connecting to the heart showed normal size and relative positioning (Fig. 3c, d). Generally, vessel wall thick-

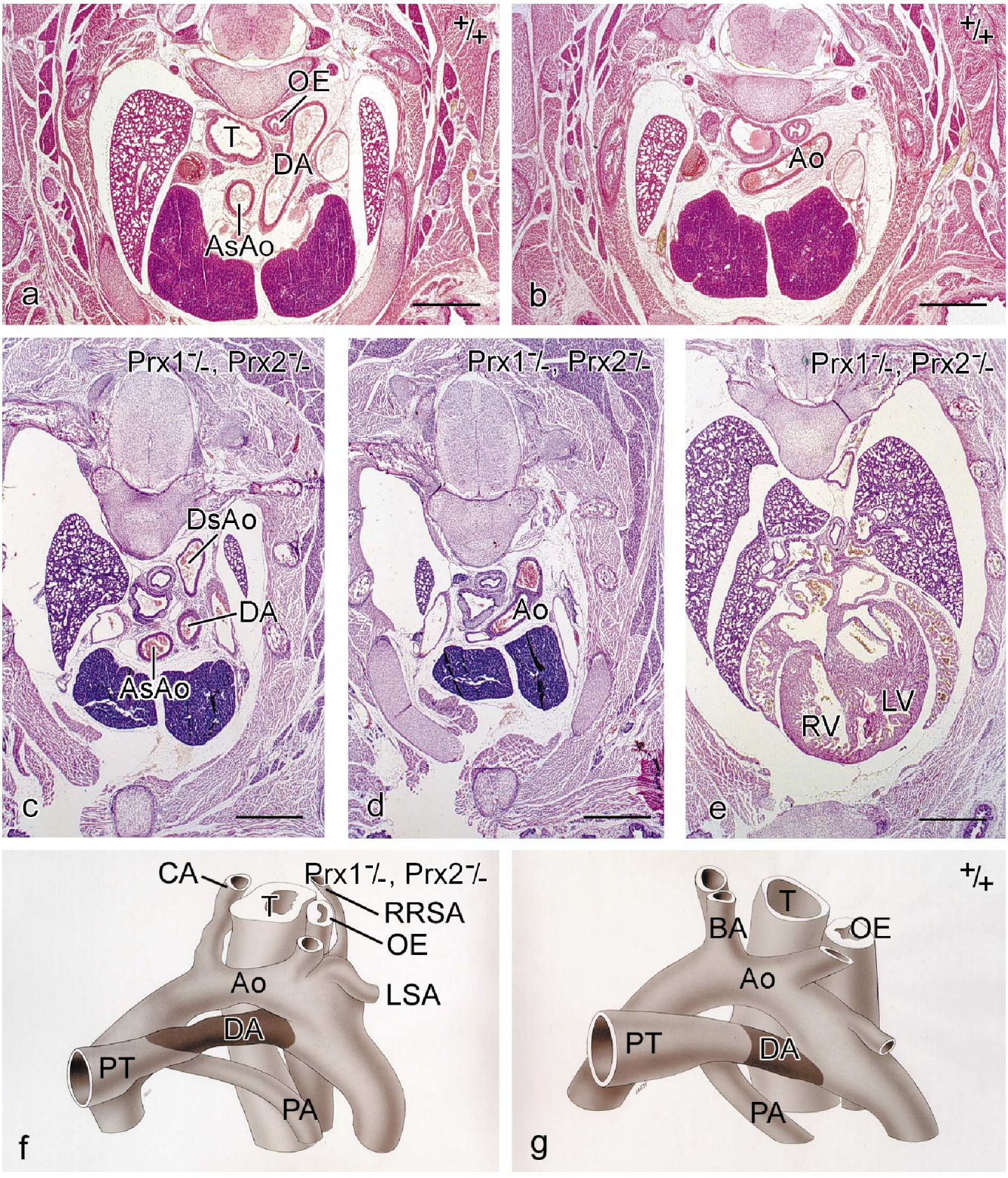

Fig. 2a–g Low-power micrographs of thoracic sections of *Prx1-/-, Prx2-/-* mice and wild types at E18.5. **a, b** Standard transverse sections through the DA (**a**) and aortic arch (**b**) in an E 18.5 wild-type mouse. Note that normally, the DA is cut longitudinally and the aortic arch runs in a straight diagonal course within the mediastinum. *Bars* 750 µm **c, d** Sections of a double mutant embryo at similar levels to those in **a** and **b**, respectively. The DA (**c**) is cut obliquely in the double mutant, and the aorta (**d**) shows a sideways curvature. *Bars* 750 µm **e** Transverse section through the heart of a double mutant, showing normal cardiac morphology, including normal atrioventricular valves. *Bar* 750 µm **f** Graphic reconstruction of the arterial tree of the double mutant shown in **c**, **d** and **e**. Note the slightly sinuous character of all great arteries, the elongated and misdirected DA, and the anomalous retro-oesophageal right subclavian artery (arteria lusoria). The typical wall of the DA was easily distinguished in sections based on haematoxylin/eosin and resorcin/fuchsin standard histological stainings and is represented in *dark shading* in the figure. **g** Graphic reconstruction of the arterial tree of the wild type

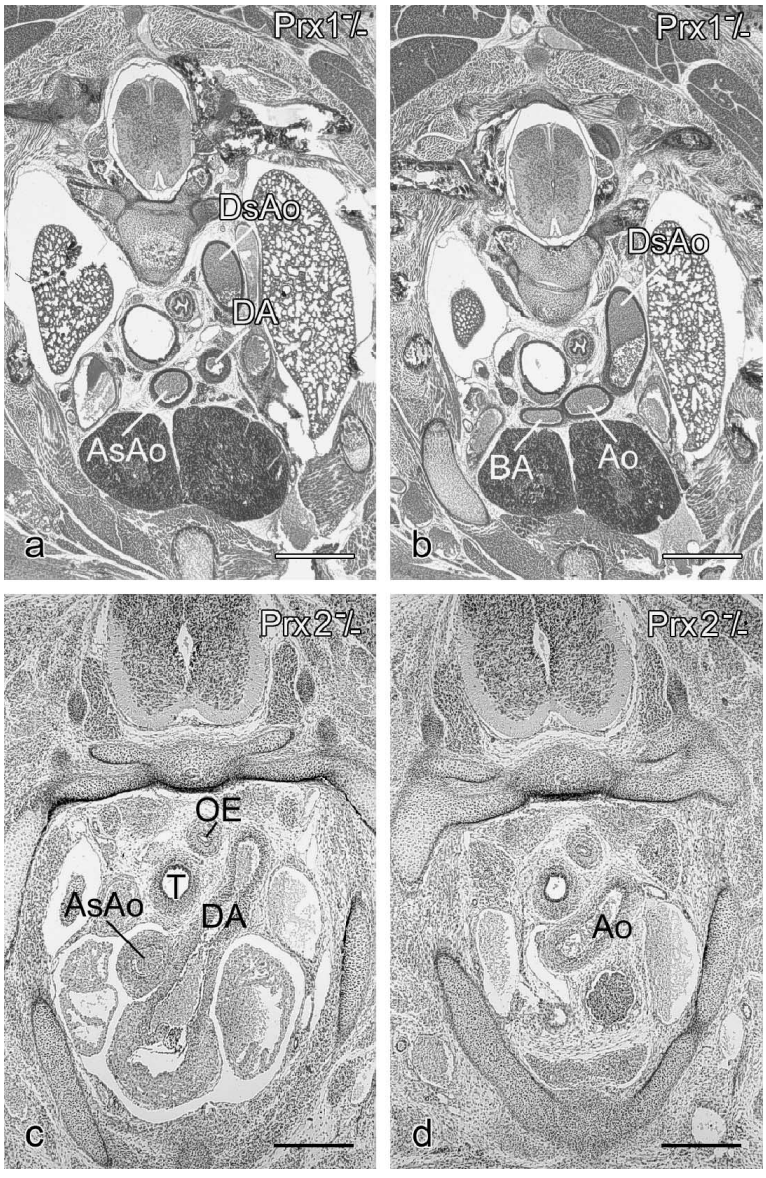

Fig. 3a–d *Prx1-/-* at E18.5, showing a phenotype very similar to the *Prx1-/-*, *Prx2-/-* double mutant (See Fig. 2c, d). **a** Section at the level of the DA. **b** Transverse section at the level of the aortic arch. *Bars* 750 µm **c, d** *Prx2-/-* at E14.5, showing normally positioned DA (**c**) and normal aortic arch (**d**). (Compare with wild type, Fig. 1e, f). *Bars* 400 µm

ness or structure did not differ from those of wild-type mice of similar developmental stages. Also, the DA, which was previously shown to express specific and high *Prx2* levels, revealed an apparently normal differentiation and was observed to undergo normal constriction and closure shortly after birth. This is consistent with the fact that *Prx2-/-* mice are completely viable and fertile (M.J. Kern, pers. commun., 1997).

Discussion

Although multiple roles have been attributed to *Prx1* and *Prx2* on the basis of molecular evidence and expression studies, their role in the development of the vascular system remains elusive. Previous studies on the expression patterns of *Prx* genes in the developing cardiovascular system suggested an important role for these genes in the development of the connective tissues of this organ system. *Prx1* was highly expressed in the endocardial cushions and later in semilunar and atrioventricular valves [15], and was furthermore reported to attain high transcript levels in both arterial and venous vessel walls [2]. *Prx2* transcripts were, in addition to moderate expression in the valves, localised in the ventricular septum and diffusely throughout the rest of the myocardium [15]. The DA was found to express high levels of *Prx2* in its media, in contrast to the other great arteries [2, 15].

In the present study, the vascular system of *Prx1* and *Prx1/Prx2* combined double-mutant mice was shown to be aberrant. The observed spectrum shows irregular, meandering vessels, a misdirected and elongated DA, and an abnormal architecture of the aortic arch. Based on the fact that deletion of *Prx*-function does not appear to lead to major alterations in the remodelling of the embryonic pharyngeal arterial system, it is unlikely that these homeodo-

main transcription factors are involved in the gross patterning of the pharyngeal arterial apparatus. In this respect, the occurrence of an anomalous retro-oesophageal right subclavian artery (RRSA, arteria lusoria, seen in 2 of 7 double mutants), may be considered a secondary rather than a primary defect. RRSA was found to be associated with many other cardiovascular anomalies in man [1]. Yet the occurrence of RRSA might also indicate that primary alterations of some kind existed in the neural crest of the early embryonic fourth pharyngeal arches, which may have affected both the aortic arch and the proximal right subclavian artery derived from them [3, 14].

In order to deduce the main *Prx* function in vascular development from the phenotype observed, one would need to speculate on what could have caused the abnormal course of the major blood vessels. Since these two genes encode transcription factors it is essential to identify the genes they regulate when determining *Prx* function.

The *Prx1* and *2* genes are very similar. For example, they encode proteins that can bind to the same sequence [7], and what is said about *Prx1* targets may also be relevant for *Prx2*. Less is known about the PRX2 protein, its potential targets, and mode of action. *Prx1* was previously suggested to be involved in regulation of matrix modulation [2]. Its pattern of expression is closely related to that of procollagen I [2]. Promoter binding and cotransfection studies are more informative and revealed that the PRX1 protein was capable of considerable repression of collagen I α1 promoter activity in vitro [11]. *Prx* loss of function in vivo may cause a misregulation of matrix composition, particularly altered procollagen I expression, thus causing alterations in developing vessel walls. Interestingly, the structures that are affected in collagen I-related disorders, like osteogenesis imperfecta, are similar to those affected in *Prx1* and *Prx1/Prx2* mutants. In both cases, skeletogenesis is most severely affected, albeit in different ways [16, 17, 22, 27]. Collagen-I disorders also include cardiovascular symptoms, such as aortic and mitral regurgitation, hypertension and dilated blood vessels [30]. *Prx-/-* mice may be susceptible to similar cardiovascular symptoms, although early neonatal death impedes both proper physiological research and examination of age-related vascular pathologies.

Prx2 mutant mice are morphologically normal, as well as completely viable and fertile. However, hearts from *Prx2* gene-targeted mice were recently shown to have slightly impaired cardiac function and altered responsiveness to a β-agonist, whereas individual cardiomyocytes were more responsive to the β-agonist. It was suggested that altered cardiomyocyte–matrix interactions may be a primary defect in these mice (M.J. Kern, pers. commun., 1997).

Recently, Hautmann et al. [10] reported on the part that *Prx1(MHox)* might play in angiotensin II-mediated expression of smooth muscle actin, thereby directly linking *Prx1* expression to contractile performance of the vessel wall. Although previous research by our group disputes a role for *Prx* genes in smooth muscle cell (SMC) contractile machinery regulation [2], further studies of mRNA and protein expression for differentiation markers of SMCs in these mice will certainly aid in elucidating the actual role of *Prx* genes. Interestingly, the angiotensin II receptor, AT_2, shows a vascular expression pattern which mimics that of *Prx1* and procollagen I [2, 26].

The phenotype of gene-targeted mutants is not always identical from mouse to mouse, even though they have the same genotype. A spectrum of skeletal malformations has been described in the *Prx1* null mutant [17] and in *Prx1/Prx2* double mutants [16, 27], and we have now demonstrated some variation in expressivity in the vascular system. Two of six *Prx1* mutant mice appeared to have a normal cardiovascular morphology. These data suggest two main possibilities. One, that stochastic or random events are also involved. Two, that genetic background may interplay with these mutant alleles and modify the phenotype. This latter possibility is particularly likely, since the *Prx1* and *Prx2* mutant alleles are on a mixed genetic background of 129 SV/J, CF-1, and C57BL/6 (M.J. Kern, pers. commun., 1997).

The vascular phenotype described in the present study appears to be caused mainly by deletion of *Prx1*, as *Prx2-/-* mice were morphologically normal. However, additional depletion of *Prx2* in the double-null mutants resulted in more severe anomalies of the vascular system. Thus *Prx2* can compensate partially for the loss of *Prx1*, whereas *Prx1* can completely overcome the deletion of *Prx2*. This *Prx1* epistasis was also observed in other regions of the developing embryo: viability, craniofacial development, inner ear, and zeugopod limb bones [16, 27]. In other tissues, *Prx2* is at least as important, if not more so [16]. This occurrence of genetic redundancy, albeit with *Prx1* epistatic over *Prx2*, raises many evolutionary questions. Cooke et al. [6] theorised on the origin and maintenance of genetic redundancy, and they elucidated some possible mechanisms which may well apply to *Prx* genes.

Despite the reported high expression levels in the connective tissues of the developing heart, no histological cardiac malformations could be detected in *Prx1*, *Prx2* or *Prx1/Prx2* combined gene-targeted mice. Valvular morphogenesis appeared normal in spite of the deletion of both genes, suggesting that either the genes are not essential for normal valve formation, or that there is functional compensation by other genes. Still, anomalies in proper haemodynamic functioning of the valves in vivo might not be reflected in their gross morphology. Clinical symptoms such as aortic and mitral regurgitation occur in patients with genetic disorders of extracellular matrix proteins and do not necessarily present clear morphological anomalies. If deleting *Prx1* alters, for instance, collagen I expression, we may not have been able to detect this by examining histological sections. In addition, the specific and high expression levels of *Prx2* from early DA differentiation to nearly birth suggested that this gene might be involved in this vessel's particular development. However, differentiation and closure of the DA are normal in *Prx2* mutant mice. As *Prx1/Prx2* combined null mutants also show no major alteration

in DA histological differentiation or postnatal closure (D. ten Berge and F. Meijlink, personal communication, 1997) and mutual compensation is herewith excluded, neither gene appears to be responsible for defining the unique DA status. Alterations in the courses of the aorta and the DA and supposedly in their length in *Prx1* and *Prx1/Prx2* combined mutants suggest that the genes have a general architectural role in all vessels. They appear to be necessary for appropriate placement of vessels in the mesenchyme via proper interaction of haemodynamics with the matrix scaffolding.

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