Defective vascular development in connexin 45-deficient mice

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SUMMARY

In order to reveal the biological function(s) of the gap-junction protein connexin 45 (Cx45), we generated Cx45-deficient mice with targeted replacement of the Cx45-coding region with the lacZ reporter gene. Heterozygous Cx45het mice showed strong expression of the reporter gene in vascular and visceral smooth muscle cells. Cx45-deficient embryos exhibited striking abnormalities in vascular development and died between embryonic day (E) 9.5 and 10.5. Differentiation and positioning of endothelial cells appeared to be normal, but subsequent development of blood vessels revealed impaired formation of vascular trees in the yolk sac, impaired allantoic mesenchymal ingrowth and capillary formation in the labyrinthine part of the placenta, and arrest of arterial growth, including a failure to develop a smooth muscle layer surrounding the major arteries of the embryo proper. As a consequence, the hearts of most Cx45-deficient embryos were dilated. The abnormal development of the vasculature in the yolk sac of Cx45−/− embryos could be caused by defective TGFβ signalling, as the amount of TGFβ1 protein in the epithelial layer of the yolk sac was largely decreased in the E9.5 Cx45−/− embryo, compared with the wild-type embryo. The defective vascular development was accompanied by massive apoptosis, which began in some embryos at E8.5 and was abundant in virtually all tissues of the embryos at E9.5. We conclude that in Cx45−/− embryos, vasculogenesis was normal, but subsequent transformation into mature vessels was interrupted. Development of different types of vessels was impaired to a varying extent, which possibly reflects the complementation by other connexin(s).

Key words: Connexin 45, Cx45, Gap junctions, Vascular development, Apoptosis, Smooth muscle cells

INTRODUCTION

Cells communicate with their neighbours in order to coordinate differentiation, growth of tissues and physiological activities. One form of communication between cells is mediated by gap junctions, i.e., clusters of channels that enable short-range signalling and propagation of electric activities by allowing the exchange of second messengers like cAMP, IP3, Ca2+ and other ions (for review see Bruzzone et al., 1996; Lo, 1996). Each gap-junction channel is formed by docking of two hemichannels located in the plasma membrane of adjacent cells. Each hemichannel consists of six subunit proteins, called connexins (Cx), that are encoded by at least 15 mouse connexin genes (White and Paul, 1999; Soehl, 1998; Condorelli, 1998; Manthey et al., 1999). Connexin 45 (Cx45; Gja7 – Mouse Genome Informatics) is a member of this multigene family. Its cDNA was isolated from F9 embryonic carcinoma cells (Hennemann et al., 1992; Haefliger et al., 1992). Subsequently, Cx45 protein has been detected immunohistochemically in many rodent tissues, including glomeruli and tubules of mouse kidney (Butterweck et al., 1994a), mouse embryonic skin (Butterweck et al., 1994b), a rat osteoblastic cell line (Steinberg et al., 1994), granulosa cells of rat ovary (Okuma et al., 1996), rat oligodendrocytes (Kunzelmann et al., 1997), in the deep muscular plexus of rat small intestine (Nakamura et al., 1998) and in motoneurons of rat spinal cord (Chang, 1999). Furthermore, Cx45 has also been reported to be expressed in rodent embryonic and adult cardiomyocytes (Alcoléa et al., 1999; Coppen et al., 1998, 1999). Whereas there is convincing evidence that Cx45 is strongly expressed in the atrioventricular (AV) node, His bundle and peripheral Purkinje fibres (Coppen et al., 1998, 1999), it remains unclear whether Cx45 is also present in the working myocardium as originally reported by (Davis et al., 1994). Alcoléa et al. (1999) observed Cx45 transcripts using in situ hybridization in the paired dorsal aortae at E10 and coronary vessels of mouse embryos at E18, but immunolabelling of coronary vessels in adult mouse heart showed only faint, infrequent signals, indicating the low immunoreactivity of Cx45-containing gap-junction plaques with most Cx45 antibodies.
During the past five years, seven connexin genes have been disrupted in the mouse genome, in order to investigate the function of the corresponding connexin proteins (reviewed in White and Paul, 1999; Willecke et al., 1999). Given the widespread expression of connexins during early embryogenesis (Lo, 1996), it was surprising that none of these connexin null mutants resulted in severe disturbances of embryonic tissue differentiation and showed very restricted or no apparent phenotypic abnormalities during embryonic development. Only the targeted inactivation of mouse Cx26 (Gjb2 – Mouse Genome Informatics) led in all cases to early embryonic lethality, probably due to defective fetomaternal transfer of nutrients (Gabriel et al., 1998). Postnatal lethality based on developmental defects has been found in Cx43-deficient mice (B. et al., 1995) that died shortly after birth owing to a cardiac malformation that leads to obstruction of the right ventricular blood outflow to the lungs (B. et al., 1995; Y. et al., 1998). As most cells express two or more connexin isoforms, it has been proposed that the loss of one connexin isotype can be partially or completely compensated by a different connexin isotype, thus limiting developmental defects and tissue malformations (cf. White and Paul, 1999 with Willecke et al., 1999).

Here we report the generation and characterization of Cx45-deficient mice that provide direct evidence for an important role of gap-junctional communication in development and differentiation. We disrupted the mouse Cx45 gene by replacing the coding region of Cx45 with the bacterial β-galactosidase (lacZ) reporter gene via homologous recombination in embryonic stem (ES) cells. Unexpectedly, we find that Cx45 is essential for vascular development. Cx45-deficient embryos exhibit defects in remodelling and organization of blood vessels after proper initiation of vasculogenesis, and fail to form a smooth muscle layer surrounding the major arteries. Accordingly, homozygous mutant mice died between E9.5 and E10.5. The defect in surrounding the major arteries. Accordingly, homozygous Cx45-deficient embryos exhibit defects in remodelling and fail to form a smooth muscle layer surrounding the major arteries. Thus, in the resulting construct pCx45HRZ-A, 663 bp of the Cx45 coding region downstream the Cx45 start codon were replaced by a promoterless lacZ reporter gene, followed by a polyadenylation signal and a neo coding region under control of the MCI promoter followed by a second polyadenylation signal.

To obtain homologously recombined embryonic stem (ES) cell clones, the targeting construct was electroporated into HM1 ES cells as described (Theis et al., 2000). Among the targeted ES cells, clones with single integration events were identified by Southern blot hybridization and injected into C57BL/6 blastocysts to obtain chimeras. In order to test for germline transmission, the chimeras were crossed with C57BL/6 mice. Cx45+/− offspring were intercrossed to test for viability of Cx45+/− mice and to expand the colony. All experiments were carried out on a mixed C57BL/6 × 129P2/OlaHsd (HM1) genetic background. Whenever possible, wild-type littermates were used as controls.

Mice were kept under standard housing conditions with a 12h-12h dark-light cycle, and with food and water ad libitum. Embryos were generated by naturally timed matings of Cx45+/− mice. Successful matings were identified by the presence of a vaginal plug on the morning after mating which was regarded as E0.5.

Genotyping of ES cells and mouse tissues
Correct targeting in ES cell clones and transgenic mice was confirmed by Southern blotting of Xhol-digested genomic DNA. The internal probe recognized a 5.7 kb Xbal fragment of the wild-type allele and a 13 kb XbaI fragment of the targeted allele.

For routine analysis, genomic DNA was prepared from tail biopsies or small pieces of yolk sacs. Allele-specific PCR was performed using the following primers: 5′-GACGGAGGTCTTCCCATCCC-3′, complementary to bp 1638 to bp 1619 of mouse Cx45 mRNA (Hennemann et al., 1992), common to both wild type and targeted locus; 5′-GATGCGTCCAATTCACCC-3′, complementary to the deleted region of the second exon of the Cx45 gene (bp 1050 to bp 1070 of mouse Cx45 cDNA (Hennemann et al., 1992)) to detect the wild-type allele; and 5′-ATATTCTGAAGCTTGCCGGC-3′, complementary to the neo cassette to detect the targeted allele.

Northern blot hybridization
Total RNA from adult tissues was collected using TRIZOL™ (Gibco BRL) according to the manufacturer’s protocol. After Northern blotting, hybridization was performed using Quick Hybridization solution (Stratagene, La Jolla, CA) at 68°C and a 32P-labeled probe, including the entire Cx45 cDNA (2074 bp).

Immunoblot analysis
Aorta and heart homogenates and lysates from HeLa-Cx45 transfected cells were subjected to standard immunoblot analysis using affinity purified rabbit anti-Cx45 (Butterweck et al., 1994a) followed by incubation with 125I-labelled protein A and autoradiography.

Histological analysis
Conceptuses, still within the uterus, or isolated yolk sacs, were fixed in 4% formaldehyde or methanol:acetone:water (2:2:1), dehydrated in a graded series of ethanol and embedded in Paraplast plus (Sherwood Medical Co., St. Louis, MO, USA). Paraffin sections (1 μm or 7 μm) were stained with Haematoxylin and Eosin or Azoploxyhine. Alternatively, implantation sites were fixed in 2.5% glutaraldehyde overnight and postfixed in 4% osmium tetroxide, dehydrated and embedded in Epon/Araldite. Semi-thin sections (0.5 μm) were stained with toluidine blue.

β-Gal staining
Whole-mount staining of embryos expressing the bacterial β-galactosidase (β-gal) reporter gene (lacZ) were carried out as described previously (Carmeliet et al., 1996a). Cryostat sections (25 μm) were fixed in 0.2% glutaraldehyde (5 minutes) or (when β-gal staining and immunohistochemistry were done on the same section) in 2%
formaldehyde (10 minutes). Staining was performed using the substrate 5-bromo-4-chloro-3-indolyl-β-galactoside (X-gal).

Immunohistochemistry
For immunohistochemistry, sections from tissues fixed with methanol:acetone:water (2:2:1) were incubated with 3% (v/v) H₂O₂ in 70% ethanol/PBS (pH 7.4) for 30 minutes to inhibit endogenous peroxidase activity, followed by incubation in TENG-T (10 mM Tris, 5 mM EDTA, 150 mM NaCl, 0.25% gelatin, 0.05% Tween-20, pH 8.0) for 30 minutes to decrease nonspecific binding. The pretreated sections were incubated overnight with monoclonal antibodies to α-smooth muscle actin (α-SMA, IMMH-2, 1:1000 diluted, Sigma, Taufkirchen), with rabbit anti-TGF beta1 (sc-146, 1:50 diluted, Santa Cruz, USA) or goat anti-TGF beta receptor type II (sc-400-G, 1:75 diluted, Santa Cruz, USA).

For immunohistochemistry combined with β-gal staining, the sections were incubated in 3% (v/v) H₂O₂ in 10% methanol/Tris-buffered saline (TBS) (pH 7.4) for 30 minutes to inhibit endogenous peroxidase activity, followed by 0.5% Triton-X100/TBS for 5 minutes to permeabilize cells and 4% bovine serum albumin (BSA)/TBS for 30 minutes to decrease nonspecific binding. The sections were then incubated overnight with rabbit anti-von Willebrand factor (1:100 diluted, DAKO, Glostrup, Denmark) or mouse monoclonal antibodies to neuronal nuclei (NeuN) (1:50 diluted, Chemikon, Temecula, USA). This was followed by incubation with appropriate secondary antibodies and rabbit peroxidase-antiperoxidase complex. Antibodies were diluted in PBS. Each incubation was followed by three washes in PBS for 5 minutes. The immuno-complexes formed were visualized by staining with 3,3'-diaminobenzidine tetrahydrochloride (DAB) (Sigma) and 3 mM H₂O₂. After staining, the sections were taken rapidly through a graded series of ethanol dilutions, cleared in xylene and mounted in Entellan (Merck, Darmstadt, Germany).

TUNEL assay
Embryos, fixed with 4% formaldehyde, were embedded in paraffin and sectioned at 7 μm. After deparaffination and hydration, the sections were pretreated with 0.3% (v/v) H₂O₂ in 70% ethanol/PBS (pH 7.4) for 30 min to inhibit endogenous peroxidase activity, followed by pre-incubation in TENG-T (10 mM Tris, 5 mM EDTA, 150 mM NaCl, 0.25% gelatin, 0.05% Tween-20, pH 8.0), supplemented with 1:100 diluted goat serum for 15 min to decrease nonspecific binding. The pretreated sections were incubated with a mixture of terminal deoxynucleotidyl transferase with label (Boehringer Mannheim, Germany) in a humidified chamber at 37°C for 90 min. This incubation was followed by incubation with 1:15 converter-peroxidase (POD), diluted in 100 mM Tris (pH 7.5) and 150 mM NaCl for 90 min in a humidified chamber at 37°C. Each incubation was followed by three washes in PBS for 5 min. The immuno-complex formed was visualized by staining with DAB (Sigma) and 3 mM H₂O₂. After staining, the sections were rapidly taken through a graded series of ethanol dilutions, cleared in xylene and mounted in Entellan (Merck, Darmstadt, Germany).

RESULTS
Targeted disruption of the Cx45 gene
A targeting construct in which 663 bp of Cx45 coding region just downstream of the start codon were replaced by a promoterless lacZ reporter gene and a neo<sup>−</sup>-cassette, was used to generate Cx45<sup>+/−</sup>-ES cell (ES) clones (Fig. 1A). Of 174 G418-resistant clones, 13 proved to be correctly recombined, as shown by Southern blot hybridization, yielding a recombination frequency of 7.5%. Injection of one clone into C57BL/6 blastocysts resulted in a chimera that transmitted the mutated allele through the germline and gave birth to viable Cx45<sup>+/−</sup>-offspring when mated with C57BL/6 females as shown by Southern blot hybridization and PCR analysis (Fig. 1B,C).

Expression of the Cx45 gene in neonatal and adult mice
The targeting construct used to disrupt the Cx45 gene contained a promotorless lacZ gene. Therefore, Cx45 gene expression could be monitored using the β-gal assay. We examined the expression pattern of the Cx45 gene in heterozygous Cx45<sup>+/−</sup> mice at E8.5, E9.5, neonatal day 2, and 2 to 4 months after birth.

Cx45 was expressed in a wide variety of neonatal and adult mouse tissues. Table 1 provides a summary of the expression pattern of Cx45. In blood vessels of these tissues, intense β-gal staining was found in the vascular smooth muscle layer; and
only occasionally weak signals were observed in the endothelium. (Fig. 2A,B). This previously unnoticed expression of Cx45 was verified by northern- and immunoblot analysis of aorta (Fig. 3A,B). Strong staining for β-gal was also observed in visceral smooth muscle cells, such as adult and neonatal intestine (Fig. 4A-C), trachea, bronchi and bronchioli, ureter, bladder (data not shown), and ductus deferens (Fig. 4D,E).

We also noticed β-gal staining in the seminiferous tubules and interstitial cells of adult testes (Fig. 4F,G). In adult Cx45+/− kidney, lacZ expression was detected in the smooth muscle layer of kidney, and in the smooth muscle layer of aorta (Fig. 2A,B). Strong staining for β-gal was also observed in visceral smooth muscle cells, such as adult and neonatal intestine (Fig. 4A-C), trachea, bronchi and bronchioli, ureter, bladder (data not shown), and ductus deferens (Fig. 4D,E).

<table>
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<tr>
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n.d., not determined; n.s., not shown.

Fig. 2. Transcriptional expression of Cx45 in adult blood vessels of Cx45+/− mice detected by β-gal staining. (A) The smooth muscle cell layer (sm) of mouse aorta is stained blue but the endothelial layer (e) is not. (B) The smooth muscle layer stained blue with X-gal is distinguished from the endothelial cell layer, which is stained reddish brown after subsequent immunostaining with anti-von Willebrand factor antibodies. Thickness of sections (A), 10 μm; (B), 20 μm. Scale bar: 50 μm.

Fig. 3. Detection of Cx45 transcript and protein by northern and immunoblot analysis. (A) Northern blot hybridization showing expression of the 2.2 kb Cx45 transcript in aortic and cardiac RNA (20 μg in each lane) in Cx45+/+ and Cx45−/− animals. (B) Immunoblot. Affinity-purified Cx45 antibodies specifically recognized a 45 kDa protein in heart and aorta homogenates. Lysate of HeLa-Cx45-transfectants (Butterweck et al., 1994a) was used as a positive control.
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Expression of Cx45 gene indicated by β-gal staining in various tissues of neonatal and adult Cx45+/− mice. β-gal staining was found in smooth muscle layers of the intestine in adult (A,B) and neonatal (C) mice. (A) is a low-magnification image of an adult small intestine; (B) is the boxed region in (A); (C) is a high-magnification image of the corresponding region in the small intestine of a neonatal mouse. (D) Smooth muscle layers of adult ductus deferens. (E) High-magnification of ductus deferens. Staining at the luminal border of the epithelium is probably nonspecific, as it is also seen in wild-type sections (not shown). (F) Adult testis. (G) Higher magnification of testis showing β-gal staining in the seminiferous tubules and interstitium. (H) Adult kidney. c, cortex; m, medulla. (I) Higher magnification, showing glomeruli (gl). (J) Boxed region in (H). (K) Adult heart with atrioventricular-node (av), right and left bundle branch (rbb, lbb) and interventricular septum (ivs). (L) Arteries and working myocardium of adult heart. (M) Skin of neonatal mouse. (N) Bronchial cartilage of a bronchus of an adult mouse. c, cartilage; l, lumen of the bronchus. (O) High-magnification of boxed region in (N). (P) Cartilage of neonatal mouse. (Q) Ossification center in the vertebral column of a neonatal mouse. (R) Higher magnification of boxed region in (Q). The sections in (A), (B) (E), (F) and (G) were counterstained with Eosin, the section in (H) and (I) was counterstained with Hematoxylin, and the sections in (J), (M), (N) and (O) were counterstained with Hematoxylin and Eosin. Thickness of all sections is 20 μm. Scale bar: 500 μm in A,F,N; 50 μm in B,C,O; 250 μm in D; 40 μm in E,R,P; 70 μm in G,I,L,L,M; 1.6 mm in H; 200 μm in K,Q.
layer of vessels, in the glomeruli (Fig. 4H,I) and in medulla
(Fig. 4H,J). Examination of serial sections from an adult mouse
heart supported the findings of Coppen et al. (1999), that
Cx45 is expressed in the AV node, His bundle and bundle branches
(Fig. 4K), but in addition showed a faint staining of some
myocytes of the working myocardium (Fig. 4L). In the
neonatal skin we found strong β-gal staining in the dermis (Fig.
4M). We observed no β-gal staining in the dermis of adult skin
(not shown). In both, neonatal and adult mice, β-gal staining
was found in cartilage (Fig. 4N,O,P), whereas the developing
bone of neonatal Cx45+/− mice, but not adult bone, showed
abundant expression of the lacZ reporter gene in osteoblasts
and osteocytes (Fig. 4Q,R).

lacZ expression was abundant in neonatal neural tissues. X-
Gal staining was particularly prominent in neonatal brain
cortex (Fig. 5A,B), but less dense staining was also detected in
all other areas of the neonatal brain (Fig. 5A). In the adult
neocortex, Cx45 expression was retained by only a few cells,
as demonstrated by the sparse β-gal staining in a subset of
neurons in the pyramidal cell layer (Fig. 5C,D). Two other
neural tissues displayed a similar downregulation of Cx45
expression from neonatal to adult stage: the retina (compare

![Fig. 5. Expression of Cx45 gene indicated by β-gal staining in various neural tissues of neonatal and adult Cx45+/− mice. Staining was seen in (A), head of neonate. (B) Higher magnification of the boxed region in (A). Cx45 expression is particularly prominent in neonatal brain cortex. (C) Neocortex of an adult mouse. (D) The higher magnification of the boxed region in (C) reveals that the blue β-gal staining and the brown immunostaining with anti-neural nuclei (NeuN) antibodies are colocalized in the soma of the same cell. (E) Higher magnification of the boxed region in (A) showing the developing retina of a neonate with prominent β-gal staining in all rows of photoreceptor cells. (F) Spinal cord of neonatal mouse. (G) The higher magnification of the boxed region in (F) shows prominent β-gal staining in the dorsal horn (dh). (H) In the corresponding area in the dorsal horn of an adult spinal cord, only a few scattered blue spots are seen. (I) Thalamus of an adult mouse. (J) Higher magnification of the boxed region in (I). Arrows point to nerve fibres stained for β-gal. (K) Neurons of a section of adult thalamus stained blue with X-gal and brown after subsequent immunostaining with von Willebrand factor antibodies. Owing to a different fixation procedure and slice thickness compared with (J) (see Materials and Methods section), the X-gal staining is only visible in the somata of neurons, presumably in areas of high abundance of β-gal protein. (L) Hippocampus of an adult mouse. The CA3/CA4 region of the hippocampus is stained with X-gal. Sections in H,J,L were counterstained with Eosin. For C,D,K, thickness of sections was 10 μm. For all other images the thickness of sections was 20 μm. Scale bar: 1mm in A; 60 μm in B; 500 μm in C,F,I; 40 μm in D,E,K; 100 μm in G,J; 60 μm in H, 240 μm in L.}
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Fig. 5E and data in Güldenagel et al., 2000) and the dorsal horn of the spinal cord (compare Fig. 5F,G and H). In sections of adult brain, we found β-gal staining specifically in a subset of neurons in the thalamus (Fig. 5J,K), in the CA3/CA4 region of the hippocampus (Fig. 5L) and, as mentioned above, in the pyramidal cell layer of the neocortex (Fig. 5C,D). A more detailed study of the Cx45 expression pattern in neural tissues and characterization of the subsets of neural cells that express Cx45 will be published elsewhere.

Expression of the Cx45 gene during early development

At E8.5, lacZ-positive cells were seen in the neural plate, notochord, somites, pericardioperitoneal canal, gut, in the branchial arches and in the outflow tract (Fig. 6A,B), the wall of AV canal and the atrium of the heart (not shown). At E9.5, staining was present at varying intensities almost everywhere in Cx45+/− embryos (Fig. 6C). In extra-embryonic tissues (Fig. 6D,E), intensive staining was seen in tissues that receive contributions from the extra-embryonic mesoderm, such as the amnion, mesodermal layer of the yolk sac, the chorionic plate, and in mesenchymal cells growing from the chorionic plate into the placental labyrinth. By contrast, the epithelial layer of the yolk sac, the placental labyrinth and spongiotrophoblast remained unstained.

Cx45−/− embryos die shortly after gastrulation

The fertility and gross appearance of mice heterozygous for the Cx45 mutant allele (Cx45+/−) were normal. However, no homozygous animals were found among 352 newborn mice from heterozygous intercrosses, indicating that disruption of the Cx45 gene was lethal under homozygous conditions (Table 1). To determine the time point at which the Cx45 mutation became lethal, embryos from F1 intercrosses were collected at different developmental stages and genotyped using PCR analysis of DNA isolated from small pieces of yolk sac. Mutant embryos were recovered at E8.5 and E9.5 with the expected Mendelian frequency (22% and 26%, respectively). Cx45−/− embryos looked macroscopically normal at E8.5, but were visibly retarded in growth and development at E9.5 (Fig. 7). No live Cx45−/− embryos were found beyond E10.5. These results indicated that the homozygous Cx45−/− mutants died between E9.5 and E10.5.

Cardiovascular defects in Cx45−/− embryos

To determine the likely cause of embryonic lethality, we analyzed the embryos from Cx45+/− intercrosses at E8.5 and E9.5, focussing on the cardiovascular system of the embryo. The hearts of Cx45−/− embryos differed from those in normal embryos in that the outflow tract and left ventricle were dilated in 1 out of 5 embryos studied at E8.5, and in 9 out of 11 embryos studied (80%) at E9.5 (Fig. 8L). The extent of dilatation was variable, but the outflow tract was involved in all cases. Furthermore, the right ventricle was underdeveloped,

Fig. 6. LacZ expression in Cx45+/− embryos at E8.5 (A) and E9.5 (B). Transverse sections of a Cx45+/− embryo at ED8.5. β-Gal staining is seen in the neural tube, branchial arches, heart, gut, somites, notochord and pericardioperitoneal canals. (C) Transverse sections of a Cx45+/− embryo at E9.5. β-Gal staining is shown at varying intensities in the whole embryo as well as in the amnion and chorionic plate, whereas staining is absent in the yolk sac epithelium, placental labyrinth and spongiotrophoblast. (D) Higher magnification reveals staining in the mesodermal layer of the yolk sac, but no staining in the epithelial layer of the yolk sac. (E) Higher magnification of the boxed region in (C) shows intensive β-gal staining in mesenchymal cells that grow from the allantoic mesoderm into the placental labyrinth, a, amnion; am, allantoic mesoderm; ba, branchial arch; cp, chorionic plate; d, decidua; da, dorsal aorta; e, embryo; eys, epithelial yolk sac; fg, foregut; l, labyrinth; m, mesenchyme; mg, midgut; mys, mesodermal yolk sac; nc, notochord; ne, neuroepithelium; nt, neural tube; oft, outflow tract; pc, pericardioperitoneal canal; rm, Reichert’s membrane; so, somite; sp, spongiotrophoblast; uv, umbilical vein; ys, yolk sac. Scale bar: 107 μm in A,B; 400 μm in C; 50 μm in D; 100 μm in E.

Fig. 7. (A) Cx45+/+ embryo at E9.5 and (B) Cx45−/− littermate. Scale bar: 500 μm.
even if the delay in the development of the Cx45-deficient embryos was taken into account.

The blood vessels of two out of five Cx45−/− embryos at E8.5 were indistinguishable from those in wild-type embryos, while the other three Cx45−/− embryos differed from controls by a decreased diameter of those parts of the dorsal aorta that drained the branchial arch arteries. (Fig. 8F,H). At E9.5, all eight Cx45−/− embryos analyzed showed narrowing or absence of the dorsal aorta (Fig. 8M), except for a normal or dilated part near the tail (Fig. 8N). Furthermore, the connection of the branchial arch arteries with the dorsal aorta was narrow or absent (not shown). One out of eight Cx45−/− embryos tested also exhibited narrowing of the dorsal aorta and heart dilatation with wall thinning (Table 2), indicating that there may be a Cx45 dose-dependent regulation of embryonic blood vessel development.

At E9.5, the intact yolk sacs of Cx45−/− mutants were pale compared with those of their wild-type littermates and vessels were not readily discernible (compare Fig. 9A with 9B). To determine the onset and cause of the vascular defect in yolk sacs, we performed histological analyses of wild-type and mutant yolk sacs at E8.5 and E9.5. Vessels formed normally in the yolk sac, as demonstrated in E8.5 specimens, although vessels seemed to be wider in some Cx45+/− embryos than in...
Defective vascular development in Cx45-deficient mice

Table 2. Genotype analyses of litters after interbreeding Cx45<sup>+/−</sup> mice

<table>
<thead>
<tr>
<th>Stage of development</th>
<th>Numbers (percentage) of embryos or mice</th>
<th>Total number of embryos tested</th>
<th>Gross appearance of Cx45&lt;sup&gt;−/−&lt;/sup&gt; embryos</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cx45&lt;sup&gt;+/+&lt;/sup&gt;</td>
<td>Cx45&lt;sup&gt;+/−&lt;/sup&gt;</td>
<td>Cx45&lt;sup&gt;−/−&lt;/sup&gt;</td>
</tr>
<tr>
<td>E3.5</td>
<td>11 (19%)</td>
<td>33 (59%)</td>
<td>14 (22%)</td>
</tr>
<tr>
<td>E8.5</td>
<td>16 (29%)</td>
<td>27 (49%)</td>
<td>12 (22%)</td>
</tr>
<tr>
<td>E9.5</td>
<td>42 (27%)</td>
<td>75 (47%)</td>
<td>41 (26%)</td>
</tr>
<tr>
<td>E10.5</td>
<td>10 (33%)</td>
<td>14 (45%)</td>
<td>8 (25%)</td>
</tr>
<tr>
<td>E11.5</td>
<td>7 (33%)</td>
<td>10 (48%)</td>
<td>4 (19%)</td>
</tr>
<tr>
<td>E12.5</td>
<td>6 (24%)</td>
<td>16 (64%)</td>
<td>3 (12%)</td>
</tr>
<tr>
<td>Adult</td>
<td>231 (34%)</td>
<td>352 (66%)</td>
<td>0</td>
</tr>
</tbody>
</table>

Fig. 10. Vascular development of Cx45<sup>+/+</sup> and Cx45<sup>−/−</sup> yolk sacs. (A) Transverse section of a Cx45<sup>+/+</sup> yolk sac at E8.5 showing definite vessel formation. The individual vessels are separated by tight attachment between the mesodermal and epithelial layer. (B) Cx45<sup>−/−</sup> yolk sac at E8.5 with a mild tendency of defective vascular formation. Broad ‘vascular spaces’ are guarded by very thin fusion lines only (arrows). (C,D) Horizontal semi-thin sections of the implantation chamber reveal defective vessel formation in the Cx45<sup>−/−</sup> (D) compared with the wild-type embryo (C), resulting in a large vascular space between the mesodermal and epithelial layer. Only some connections between the two layers are observed (arrows) as signs of formation of vascular walls. (E) Semi-thin section of the wild-type yolk sac reveals intact blood vessels containing numerous embryonic blood cells. (F) In the Cx45<sup>−/−</sup> yolk sac, a continuous vascular space was formed between the two yolk sac layers. Only a few blood cells could be detected within the two layers and outside the yolk sac. a, amnion; eys, epithelial yolk sac; mys, mesodermal yolk sac; rm, Reichert’s membrane. Scale bar: 50 μm in A,B; 300 μm in C,D; 40 μm in E,F.
controls (Fig. 10A,B). However, at E9.5, severe disturbances in vascular development within the Cx45+/− yolk sac had become obvious (Fig. 10D,F). A vascular lumen with endothelium was present (Fig. 10F), but only occasionally a connection between the two layers of the yolk sac was seen as a sign of formation of the walls of a vascular tree (Fig. 10F).

As a result the vascular structure between the epithelial and mesodermal layer of the yolk sac could best be described as a large ‘vascular space’ (Fig. 10D,F). Only few blood cells were detected in this vascular space, possibly owing to the reduced level of haematopoiesis in the yolk sac. The defect in the yolk sac was consistent with the expression of Cx45 in the mesodermal layer of the yolk sac, as monitored by β-gal staining (see above, Fig. 6D).

A comparison of E9.5 wild-type and mutant placentas revealed abnormal vessel formation in this extra-embryonic structure as well (Fig. 11). During placental development in rodents, the allantois fuses with the chorion, an event that induces the ectoplacental cone cells to differentiate into the labyrinthine layer, the spongiotrophoblast layer and giant cells. This initial differentiation event seemed to be normal in Cx45 mutants. At E9.5, the labyrinth region in the wild-type placenta contained a network of extra-embryonic capillaries interspersed with maternal sinuses (Fig. 11A,C). In the Cx45−/− placental labyrinth, embryonic vessels were abnormally enlarged, while the degree of branching of the embryonic vessels was reduced. Invasion of maternal sinuses was also impaired in Cx45−/− embryos (Fig. 11D), so that the labyrinth retained a more compact appearance (Fig. 11B). In wild-type placentas, the ingrowing blood vessels caused the trophoblast

**Fig. 11.** Morphology of Cx45+/+ and Cx45−/− placentas. (A) Section of the placenta from a Cx45+/+ embryo at E9.5, and (B) from a Cx45−/− embryo at E9.5, respectively. (C,D) The boxed areas of (A,B), respectively. The vessel formation within the chorionic plate is normal in both Cx45+/+ (A) and Cx45−/− placentas (B), but invasion of embryonic vessels and maternal sinuses into the labyrinth region of the placenta is impaired (B). (C) The labyrinth of Cx45+/+ placenta shows normal capillary formation (arrows), but fewer and enlarged embryonic blood vessels (arrows) were seen in Cx45−/− placenta (D). (E) Semi-thin section demonstrating normal capillary formation in the wild-type placenta. The elongated appearance of trophoblast cells indicate the beginning differentiation of trophoblasts into syncytial layers around embryonic vessels (arrowheads). The endothelial cells of embryonic vessels are separated by matrix material from the trophoblast layer (arrow). (F) In Cx45−/− placenta, ingrowth as well as expanding and branching of the vessels into the labyrinth are decreased. Elongated trophoblasts along the embryonic vessels are missing (arrowheads). The endothelial cells are in direct contact with the trophoblast cells (arrow). ev, embryonic vessel; m, maternal sinus. Scale bar: 140 μm in A,B; 50 μm in C,D; 25 μm in E,F.
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cells around the embryonic vessels to elongate their shape and to start with the formation of two syncytial layers around the embryonic vessels (Fig. 11E). These elongated trophoblast cells were missing around the vessels of Cx45\(^{-/-}\) placentas (Fig. 11F). In Cx45-deficient embryos, we also noted that embryonic endothelial cells were in direct contact with trophoblast cells (Fig. 11F), whereas in wild-type placentas, embryonic vessels were separated by matrix material from the trophoblast layer (Fig. 11E).

The yolk sac vessels in mouse mutants lacking TGF\(\beta\)-receptor 2 (Oshima et al., 1996) or TGF\(\beta\)1 (Dickson et al., 1995) had a phenotypically similar defect to those found in Cx45\(^{-/-}\) embryos. In order to investigate whether the transforming growth factor \(\beta\) (TGF\(\beta\)) pathway is affected in Cx45\(^{-/-}\) embryos, we stained microscopic sections from Cx45\(^{+/+}\) embryos and Cx45\(^{-/-}\) embryos immunohistochemically for the presence of TGF\(\beta\)1 and TGF\(\beta\) receptor 2 protein. In the wild-type embryo, at E8.5 and E9.5, TGF\(\beta\)1 was found in the myocardium and the epithelial layer of the yolk sac (Fig. 12A,C), but not in the vessel wall (not shown). Cx45\(^{-/-}\) embryos at E8.5 showed the same pattern of TGF\(\beta\)1 protein expression as wild-type embryos (not shown). However, in Cx45\(^{-/-}\) embryos at E9.5, the amount of TGF\(\beta\)1 was decreased in both the myocardium and the epithelial layer of the yolk sac (Fig. 12B,D). In the myocardium, the expression of TGF\(\beta\) receptor 2 protein correlated with that of TGF\(\beta\)1 in Cx45\(^{+/+}\) embryos at E8.5 and E9.5. We found a downregulation of the TGF\(\beta\) receptor 2 protein similar to that of TGF\(\beta\)1 in the myocardium of Cx45\(^{-/-}\) embryos (not shown). In both, wild-type and Cx45\(^{-/-}\) embryos, TGF\(\beta\) receptor 2 was present in the epithelial layer of the yolk sac at early and late E8.5, the time when the first abnormalities in vessel diameter became visible in Cx45\(^{-/-}\) embryos. However, at E9.5, the TGF\(\beta\) receptor 2 protein was downregulated both, in wild type and

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**Fig. 12.** Expression of TGF\(\beta\)1 in embryonic heart and yolk sac. Whereas TGF\(\beta\)1 is expressed on E9.5 in the myocardium of the whole heart (A) and the epithelial layer of the yolk sac (C) of the wild-type embryo, it is not detected in the myocardium (B) and is considerably decreased in the yolk sac epithelial layer (D) of the Cx45\(^{-/-}\) embryo. at, atrium; avc, atrioventricular canal; eys, epithelial yolk sac; hg, hindgut; mys; mesodermal yolk sac; oft, outflow tract; vt, ventricle. Scale bar: 100 \(\mu\)m in A,B; 40 \(\mu\)m in C,D.

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**Fig. 13.** Expression of \(\alpha\)-smooth muscle actin (\(\alpha\)-SMA) in embryonic vasculature. In the Cx45\(^{+/+}\) embryo, \(\alpha\)-SMA is seen in the wall of the dorsal aorta (A, cranial; C, caudal) and umbilical artery (E). This contrasts with the Cx45\(^{-/-}\) embryo, where \(\alpha\)-SMA is missing in the dorsal aorta (B, cranial; D, caudal) and umbilical artery (F). Both Cx45\(^{+/+}\) (E) and Cx45\(^{-/-}\) (F) embryos show \(\alpha\)-SMA expression in the umbilical vein. In the yolk sac of the Cx45\(^{-/-}\) embryo (H), some unevenly distributed \(\alpha\)-SMA-expressing cells are seen under the epithelial layer (arrows), whereas in the yolk sacs of the Cx45\(^{+/+}\) embryo (G), the entire wall of large vessels is lined with \(\alpha\)-SMA-positive cells. da, dorsal aorta; eys, epithelial yolk sac; hg, hindgut; mys; mesodermal yolk sac; nt, neural tube; oft, outflow tract; ua, umbilical artery; uv, umbilical vein; vys, vessel of yolk sac. Scale bar: 250 \(\mu\)m in A,C; 125 \(\mu\)m in E,F; 100 \(\mu\)m in B,D,G,H.
Cx45−/− embryos (not shown). It was also undetectable in the vessel wall at these developmental stages.

As Cx45 is abundantly expressed in the smooth muscle layer of Cx45+/− blood vessels, we wondered whether this layer was formed normally in Cx45−/− embryos. We therefore stained embryos for the presence of α-smooth muscle actin (αSMA). At E9.5, Cx45+/− embryos showed αSMA-positive cells in the wall of larger arteries (Fig. 13A,C,E), the umbilical vein (Fig. 13C,E) and the large vessels of the yolk sac (Fig. 13G). By contrast, in Cx45-deficient embryos αSMA was missing along the whole length of the wall of the dorsal aorta (Fig. 13B,D) and of the umbilical artery (Fig. 13F), including a cranial segment of the aorta known to show smooth muscle differentiation in the wild-type dorsal aorta at E8.5 (Li et al., 1999) (Fig. 13B). However, we did find αSMA-positive cells in the wall of the umbilical vein of Cx45−/− embryos (Fig. 13F), where smooth muscle differentiation was previously reported to occur later than in the dorsal aorta and umbilical artery (Takahashi et al., 1996). These findings argue against a lack of αSMA expression in arteries of Cx45−/− embryos due to their general developmental retardation by half a day. Instead, they suggest a different effect of Cx45 deficiency on the development of arteries and veins. In Cx45-deficient yolk sacs, αSMA-positive cells were inhomogeneously distributed along endothelial cells and located predominantly in the region near the chorionic plate (Fig. 13H).

**Defective vascular development in Cx45−/− embryos was accompanied by massive apoptosis**

Cx45−/− embryos at E9.5 showed massive apoptosis, as demonstrated by histological observations and confirmed by TUNEL assay. Two of five embryos at E8.5 showed the prominent blebbing phenomenon, an early sign of apoptosis, and some apoptotic bodies in the cranial ganglia, branchial arch, and somites (Fig. 14B,D). Apoptosis became prominent almost everywhere in Cx45−/− embryos at E9.5 (Fig. 14F,G). The placenta of E9.5 embryos also exhibited signs of apoptosis in the mesenchymal component (not shown). In accordance, the TUNEL assay was strongly positive (Fig. 14H). The defects found in Cx45-deficient embryos at E8.5 and E9.5 are summarized in Table 3.

**DISCUSSION**

This report provides the first demonstration that gap junctional communication is necessary for normal vascular development. The formation of blood vessels, and their subsequent remodelling to cope with the demands of a growing embryo, requires extensive interactions of endothelial cells with themselves, the extracellular matrix and mesenchymal cells. Communication between cells is necessary to coordinate these interactions. Signalling molecules up to 1 kDa molecular mass can pass through gap junction channels and convey information to adjacent cells. Therefore, the extracellular matrix is an important component of cell communication during vascular development. Cx40 (Gja5 – Mouse Genome Informatics) and Cx37 (Gja4 – Mouse Genome Informatics) were abundantly expressed in the smooth muscle layer of the umbilical vein of Cx45+/− embryos (not shown). It was also undetectable in the ED 8.5 ED 9.5

<table>
<thead>
<tr>
<th>Phenotypic Abnormality</th>
<th>ED 8.5</th>
<th>ED 9.5</th>
<th>ED 8.5</th>
<th>ED 9.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth retardation</td>
<td>0/5</td>
<td>0/11</td>
<td>11/11</td>
<td>4/23</td>
</tr>
<tr>
<td>Apoptotic cell death</td>
<td>2/5</td>
<td>0/2</td>
<td>11/11</td>
<td>0/8</td>
</tr>
<tr>
<td>Heart dilation with thin walls</td>
<td>1/5</td>
<td>0/2</td>
<td>9/11</td>
<td>1/8</td>
</tr>
<tr>
<td>Narrowing of dorsal aorta</td>
<td>3/5</td>
<td>0/2</td>
<td>8/8</td>
<td>1/8</td>
</tr>
<tr>
<td>Defective formation of blood vessels in yolk sac</td>
<td>3/3</td>
<td>n.d.</td>
<td>7/7</td>
<td>0/2</td>
</tr>
<tr>
<td>Decrease of haematopoietic cells</td>
<td>1/3</td>
<td>n.d.</td>
<td>7/7</td>
<td>0/2</td>
</tr>
<tr>
<td>Decreased formation of embryonic capillaries in placenta</td>
<td>n.d.</td>
<td>n.d.</td>
<td>6/7</td>
<td>0/2</td>
</tr>
</tbody>
</table>

Numbers represent phenotypically abnormal embryos per total number of embryos analyzed.

n.d., not determined.

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Fig. 14. Extensive apoptotic cell death in Cx45-deficient embryos. Transverse section at E8.5 of wild-type embryo (A) and Cx45−/− embryo (B). (C) and (D) illustrate the boxed areas in (A) and (B), respectively, at higher magnification. The mesenchymal cells in the cranial ganglia show the prominent budding phenomenon of the cell membrane with cell shrinkage and nuclear fragmentation. (E) and (F) show a transverse section of wild-type embryo and Cx45−/− embryo at E9.5, respectively. (G) is an enlargement of the boxed area in (F). Cell death with apoptotic bodies was found almost everywhere. (H) TUNEL assay shows positive staining of the fragmented nuclei. ba, branchial arch; cr, cranial ganglia; nt, neural tube; oft, outflow tract; ot, otic vesicle. Scale bar: 75 μm in A,B; 10 μm in C,D; 350 μm in E,F; 40 μm in G,H.
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Role of Cx45 in vascular remodelling

De novo differentiation of angioblasts into endothelial cells and the formation of primitive blood vessels from angioblasts at or near the site of their origin are two distinct steps during the onset of vascularisation that are defined as vasculogenesis (Risau and Flamme, 1995). Our histological analysis of Cx45 deficient embryos has revealed that vasculogenesis was not affected, as blood vessels were initially formed as in wild-type embryos. Instead, Cx45-mediated communication was apparently necessary for the subsequent stage of vascular development, i.e., the remodelling of the initial vascular system into a tree of appropriately sized mature vessels. As a predominant feature of Cx45−/− embryos, the blood vessel diameters were abnormal in all three independently arising embryonic blood vessel systems (i.e., vasculature of yolk sac, placenta and embryo proper).

In the yolk sac of Cx45−/− embryos, we observed large endothelium-lined vascular spaces with only some residual connections between the yolk sac layers, instead of the branching network of vessels present in the wild-type embryo. Apparently, endothelium was normally laid down but the differentiation of vessels was severely compromised. The defective yolk sac vessels in Cx45−/− embryos were phenotypically similar to those found in mouse mutants lacking TGFβ receptor 2 (Oshima et al., 1996) or tissue factor (Carmeliet et al., 1996b), and in 50% of mouse mutants lacking the gene for TGFβ1 (Dickson et al., 1995). These mice also died between E9.5 and E11 but, in contrast to Cx45−/− embryos, the intra-embryonic cardiovascular system developed normally. This implies the existence of different or additional signalling pathways regulating intra-embryonic and extra-embryonic vascular development.

TGFβ signalling has been shown to regulate production and deposition of fibronectin in the extracellular matrix of the embryonic yolk sac, and it is necessary for the maintenance of yolk sac integrity as well as the formation of robust vessels by endothelial cells (Goumans et al., 1999). We show here that the amount of TGFβ1 protein in the epithelial layer is decreased in the Cx45−/− embryo at E9.5, compared with the wild-type embryo. Therefore, we conclude that the abnormal development of the vasculature in the yolk sac of Cx45−/− embryos could be caused by defective TGFβ signalling and that Cx45 is likely to be required for the maintenance of TGFβ1 expression. In the yolk sac, however, there is no detectable difference in expression of the TGFβ receptor 2 protein in the wild-type and Cx45−/− embryos. The expression of TGFβ1 receptor protein was downregulated between E8.5 and E9.5 in both wild-type and Cx45−/− embryos. We cannot exclude that the expression of the TGFβ1 receptor protein is also influenced by Cx45 during the remodelling phase of the yolk sac vasculature, as we might have missed a small time window during which the TGFβ1 receptor protein might have been downregulated earlier in Cx45−/− embryos compared with the wild-type embryos. In the myocardium, where the TGFβ1 receptor protein is still present at E9.5 in the wild-type embryos, expression of this protein was affected by the presence of Cx45.

Cx45−/− embryos revealed additional abnormalities in their intra-embryonic vasculature. These defects may have contributed to embryonic death. At E9.5, the dorsal aortae in most sections of Cx45 deficient embryos had such a small lumen that they could be hardly discriminated from surrounding tissues. In contrast, dorsal aortae were dilated in the caudal part. Inhomogeneous abnormalities in vessel structure have been also reported for Mef2c-deficient mice (Bi et al., 1999; Lin et al., 1998). Similar to our Cx45−/− mice, the two yolk sac layers were separated, and the dorsal aortae were absent or malformed in the rostral part of the Mef2c−/− embryo and dilated in the caudal part.

Defects in differentiation of smooth muscle cells in Cx45−/− mice

We also observed that the smooth muscle cell layer did not develop in the arteries of Cx45−/− embryos but did so in the umbilical veins. Smooth muscle cell differentiation in the normal mouse embryo was previously reported to occur later in the umbilical vein than in the dorsal aortae and umbilical arteries (Takahashi et al., 1996). This finding indicates that loss of Cx45 affects the development of arteries more severely than that of veins. It is likely that Cx45 affects a signalling pathway that specifically regulates the differentiation of arterial smooth muscle cells, or, alternatively, Cx45 may be involved in the differentiation of all smooth muscle cells, but its function can be taken over by another connexin(s) expressed in veins, whereas loss of Cx45 cannot be compensated for in arteries.

Concomitant with the absence of proper vessel formation, we observed in the yolk sac an inhomogeneous distribution of...
αSMA-expressing cells. We propose that lack of proper development of smooth muscle cells or pericytes contributed to the abnormal development in the yolk sac, possibly because appropriate modelling of vessel structures requires signalling of supporting αSMA-expressing cells.

Vascular defects in the placenta
In the Cx45/−/− placenta, normal blood vessel formation is also impaired, leading to fewer and enlarged blood vessels that are reminiscent of the changes seen in the yolk sac. Nutritional interaction with maternal blood circulation mediated by the chorioallantoic placenta becomes essential beyond E9.5, as embryos defective in chorioallantoic circulation, i.e., Mas2/−/− (Ascl2−/− – Mouse Genome Informatics) embryos, die around E10 (Guillemot et al., 1994). In Cx45/−/− embryos, the fetomaternal exchange of gases and nutrients is likely to be severely impaired, as there is only little contact between maternal and embryonic vessels due to the abnormal enlargement of embryonic capillaries and the impaired invasion of embryonic vessels and maternal sinuses. The nutritional interaction should be further decreased by the failure of trophoblasts to differentiate into syncytiotrophoblast layers that mediate the transport of nutrients from maternal sinuses to the embryonic blood vessels. As a consequence, the described placental abnormalities of Cx45/−/− embryos might contribute to embryonic death.

Cardiac defects and apoptosis: primary or secondary effects of missing Cx45?
The pronounced and widespread effects of Cx45 deficiency on vascular development make it difficult to discriminate primary effects of this gap junction protein from secondary effects caused by defective vascular development. Accordingly, the thinning of the ventricular wall and cardiac dilatation that we observed in Cx45-deficient embryos, could be a primary defect, as Cx45-containing channels might coordinate the very early peristaltic movements of the primitive tubular heart and loss of Cx45 could result in impairment of electrical conductance. Alternatively, these defects could be secondary to obstruction of blood flow by the abnormally narrow dorsal aortae. In fact, thinning of the ventricular myocardium has been found in mouse mutants that exhibited defects in vascular development caused by deficiency of vascular endothelial growth factor (VEGF) (Carmeliet et al., 1996a; Ferrara et al., 1996) and by disruption of the endoglin gene (Arthur et al., 2000).

Similarly, apoptosis could be a direct consequence of the loss of Cx45, or could result from deficient blood circulation. In other targeted null mutant mice that exhibited vascular abnormalities, including the Flk1/−/− (Kdr−/− – Mouse Genome Informatics) (Shalaby et al., 1995), Tgfb1−/− (Dickson et al., 1995), Vegfa−/− (Carmeliet et al., 1996a); (Ferrara et al., 1996), tissue factor−/− (Carmeliet et al., 1996b), Junb−/− (Schorpp-Kistner et al., 1999) and Smad5−/− mice (Madh5−/− – Mouse Genome Informatics) (Yang et al., 1999), increased cell death occurred after E8.5, and usually not before E9.5. Clearly, it is necessary to elucidate the function of Cx45 in early embryonic development to address these questions.

Conclusion
Homoygous disruption of the Cx45 gene leads to an interruption of vascular development after normal initiation of vessel formation and impairs differentiation of smooth muscle cells to a different extent in different blood vessels. This is a surprising discovery, as, so far, Cx45 channels were expected to function primarily in cardiac conduction. Our findings suggest that Cx45-containing gap junction channels regulate cell-cell interactions during vascular development, possibly together with other connexin protein(s). In the light of defects described above, it is conceivable that growth retardation and death of Cx45/−/− embryos between E9.5 and E10.5 could be attributed to a failure in blood circulation and impaired placental function.

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REFERENCES


