Peripheral lymphangiogenesis in mice depends on ectodermal connexin-26 (Gjb2)

Nikolai Dicke¹, Nicole Pielensticker¹, Joachim Degen¹, Julia Hecker¹, Oliver Tress¹, Tobias Bald¹, Alexandra Gellhaus², Elke Winterhager²,* and Klaus Willecke¹,*,‡,§

¹Institute of Genetics, Division of Molecular Genetics, University of Bonn, 53117 Bonn, Germany
²Institute of Molecular Biology, University of Essen-Duisburg, 45122 Essen, Germany
*These authors contributed equally to this work
†Present address: LIMES Institute, University of Bonn, Germany
‡Author for correspondence (k.willecke@uni-bonn.de)

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Summary
In order to study the specific function of connexin-26 (Cx26, also known as gap junction beta-2 protein; Gjb2), we generated knockin mice that expressed either a floxed lacZ reporter or, after Cre-mediated deletion, connexin-32 (Cx32)-coding DNA, both driven by the endogenous Cx26 promoter. Heterozygous Cx26KICx32 embryos developed normally until embryonic day 14.5 but died before birth with severe lymphedemas. Although the jugular lymph sacs were normally developed, these embryos had a strongly reduced dermal lymphatic capillary network. By analyses of β-galactosidase reporter protein expression and lymphatic or blood endothelial-specific marker proteins, we demonstrated that Cx26 expression is temporally closely linked to lymphangiogenesis. No obvious phenotypic abnormalities were observed in Cx26KICx32 mice when Cre-mediated recombination was directed to mesenchyme or blood endothelium using the Prx1–Cre or Tie2–Cre mouse strains, respectively. By contrast, keratin-5–Cre-mediated replacement of Cx26 with Cx32 or deletion of both Cx26 alleles revealed severe lymphedemas similar to the general Cx26KICx32 phenotype. Thus, conditional ablation of Cx26 (loss of function) in ectoderm leads to partial disruption of lymphatic capillaries and embryonic death. We conclude that appropriate development of dermal lymphatic vessels in mice is dependent on the expression of Cx26 in the ectoderm.

Key words: Cx26, Cx32, Ectoderm, Lymphedema, Lymphatic development

Introduction
Cellular interactions are necessary for the organization of cells into multilayered tissues or organs during development and adulthood. Connexin-26 (Cx26, also known as gap junction beta-2 protein; Gjb2) and connexin-32 (Cx32/Gjb1) are members of the connexin family that codes for 20 different proteins in the mouse genome (Söhl and Willecke, 2003). Connexins oligomerize to hexameric connexons (hemichannels). Docking and opening of two connexons result in conduits that directly connect the cytoplasm of adjacent cells for diffusional exchange of small molecules up to 1.8 kDa molecular mass (Neijssen et al., 2005). Ablation of a connexin isoform (knockout or null mutation), certain point mutated connexin isoforms or exchange of different connexin isoforms (knockin; KI) can cause strong developmental defects and embryonic death (Krüger et al., 2000; Dobrowolski et al., 2009; Frank et al., 2010). In hepatocytes, Cx26 and Cx32 proteins are largely colocalized and occur together in gap junctions (Nicholson et al., 1987). Homozygous Cx26 knockout (KO) mice are early embryonically lethal [embryonic day (E) 9.5–10.5], because Cx26 is an essential subunit protein of gap junction channels for the exchange of metabolites between maternal and embryonic blood circuits in the placental labyrinth (Gabriel et al., 1998). We wanted to investigate whether Cx32 can functionally replace Cx26 during development and in adult liver. Surprisingly, we found that the monoallelic exchange of Cx26 by Cx32 in mice did not impair placental function but led to embryonic death between E16.5 and 18.5. The mutant embryos exhibited severe lymphedemas, suggesting malformation of lymphatic vessels. Thus we decided to study this unexpected phenotype in more detail.

The lymphatic system mediates tissue fluid homeostasis, immune surveillance and fat absorption, and has been shown to be involved in the pathogenesis of several human diseases. Nearly every tissue or organ system is pervaded by lymphatic vessels, with the exception of the central nervous system, bone marrow, cartilage, cornea and epidermis (Mäkinen et al., 2007; Karpanen and Alitalo, 2008). In contrast to the circulatory cardiovascular system, the lymphatic system is a unidirectional structure, built up by blind-ending lymphatic capillaries, precollection vessels and larger connecting vessels, which are interrupted by lymph nodes. These larger lymphatics are finally connected via the thoracic duct and the right lymphatic duct to the venous blood vessel system (Tammela et al., 2005). The lymphatic system develops after the establishment of the cardiovascular system. In mammals it has been found that the whole lymphatic endothelium exclusively originates from a subpopulation of venous cells, which give rise to the total lymphatic endothelium by centrifugal sprouting (Srinivasan et al., 2007). In birds and tadpoles, the lymphatic vasculature is of dual origin: lymphatic endothelial cells arise by transdifferentiation from venous endothelial cells and by differentiation of mesenchymal lymphangioblasts (Wiltert et al., 2006; Ny et al., 2005). Recent data suggest a dual origin of lymphatic endothelial cells also in mice (Buttler et al., 2006; Buttler et al., 2008).

Several proteins have been shown to fulfill specific functions during lymphangiogenesis. For detailed reviews see Tammela et
al. (Tammela et al., 2005), Mäkinen et al. (Mäkinen et al., 2007), Tammela and Alitalo (Tammela and Alitalo, 2010) and Oliver and Srinivasan (Oliver and Srinivasan, 2010). The transcription factor Prox1 is expressed in a subpopulation of endothelial cells and has been shown to be essential for the first cells that are committed to the lymphatic endothelial cell lineage on E10. Mice in which Prox1 is ablated, fail to develop the first lymphatic structures, the jugular lymph sacs, and exhibit severe lymphedemas on E14.5 (Wigle and Oliver, 1999). Endothelial cells that are committed to the lymphatic lineage also express the lymphatic hyaluronan (HA) receptor (LYVE-1) and the vascular endothelial growth factor receptor 3 (VEGFR-3) (Banerji et al., 1999; Wigle et al., 2002). Furthermore, vascular endothelial growth factor C (VEGF-C), a ligand of VEGF-3, is required for polarized sprouting of the first lymphatic endothelial cells from embryonic veins. The authors suggested that the paracrine VEGF-C signal constitutes a concentration gradient towards which the first lymphatic endothelial cells migrate to form jugular lymph sacs (Karkkainen et al., 2004). VEGF-C knockout mice also lack the jugular lymph sacs (Karkkainen et al., 2004). Inhibition of VEGF-C and VEGF-D by expression of soluble VEGFR-3 under control of the keratin 14 promoter led to moderate lymphedemas due to regression of the dermal lymphatic network (Mäkinen et al., 2001).

Less is known about connexin functions in lymphangiogenesis and lymph vessel integrity. Recently it has been shown that certain mutations in the human gap junction protein Cx47 can cause inherited human lymphedema (Ferrell et al., 2010; Ostergaard et al., 2011). In this study we investigated the essential role of ectodermally expressed Cx26 for peripheral lymphangiogenesis in mice.

### Results

#### Generation of transgenic mice

We generated mice in which the coding region of Cx26 was replaced by a β-galactosidase (β-gal) coding region, flanked by loxP sites, preceded by a nuclear localization signal and followed by the coding region of Cx32 (Fig. 1A). The targeting vector was designed to express, under control of endogenous Cx26 regulatory elements, either lacZ- or, after Cre recombinase-mediated deletion, Cx32 coding DNA. Thus, we could analyze Cx26 expression by use of the lacZ reporter gene (in Cx26+/loxlacZ/Cx32) mice or, alternatively, we were able to study the replacement of Cx26 with Cx32 (in Cx26+/Cx32 mice; Fig. 1A). Mice were genotyped by polymerase chain reaction (PCR; Fig. 1B). Southern blot hybridizations, using external and internal probes, demonstrated homologous recombination in mice (Fig. 1C). Transgenic Cx26+/loxlacZ/Cx32 mice were viable and did not show any phenotypic abnormalities. They were used to study reporter gene expression and for breeding with different Cre-recombinase-expressing mouse lines. As overview, all the mouse mutants analyzed in this study are summarized in supplementary material Table S1.

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**Fig. 1. Generation of conditional Cx26knockin/Cx32 mice.** (A) Scheme of homologous recombination of the targeting vector into the coding region of Cx26. The resulting transgenic allele (Cx26loxlacZ/Cx32) includes a nuclear location signal (nls) fused to the lacZ coding DNA, which is flanked by loxP sites, followed by Cx32 coding DNA and a neomycin selection cassette, which is flanked by flt sites. FLP recombinase activity causes deletion of the neomycin selection cassette (Cx26loxlacZ/Cx32). After Cre-mediated deletion of the lacZ reporter DNA, Cx32 is driven by the endogenous Cx26 promoter (Cx26loxlacZ/Cx32neo). (B) PCR products specific for wild-type and transgenic loci using DNA from embryonic yolk sac tissues. (C) Homologous recombination was confirmed by Southern blot hybridization of BglII-digested genomic DNA prepared from embryos (E15.5) using an external 5′ Cx26 probe and an internal Cx32 probe. Fragment sizes of each genotype are indicated in the scheme.
Heterozygous Cx26KICx32 embryos exhibit lymphedemas

For ubiquitous expression of Cx32 instead of Cx26, we crossed Cx26+/floxed/lacZ[Cx32] mice with phosphoglycerate kinase (PGK)–Cre-expressing mice (Lallemand et al., 1998), in order to obtain heterozygous Cx26KICx32 mice (Cx26+/Cx32). Surprisingly, we did not find any postnatal live mice of the Cx26+/Cx32 genotype. Hence, we focused on embryogenesis and found that Cx26+/Cx32 embryos died during development with severe malformations (Fig. 2). However, embryonic death was not due to malnutrition of the embryo caused by transplacental transport defects as previously found with Cx26KO embryos on E10.5 (Gabriel et al., 1998), because Cx26+/Cx32 embryos were not growth retarded and died later, between E16.5 and 18.5, as a result of fatal malformations. Until E14.5 no macroscopic malformations of Cx26+/Cx32 embryos were found (Fig. 2A,B) but mutants beyond E14.5 exhibited severe tissue swelling and agnathia (Fig. 2A–F). The inner organs developed normally until E16.5, but the surrounding mesenchymal tissues had undergone severe swelling (Fig. 2B,D,F). Higher magnification revealed big intercellular spaces between the mesenchymal cells relative to controls, pointing to accumulation of fluid in the matrix (Fig. 2F; supplementary material Fig. S1). The epidermis appeared disturbed in body regions next to strong accumulation of fluid, whereas in regions where less tissue swelling occurred it was stage specifically differentiated, e.g. in caudal regions (supplementary material Fig. S1). The epidermis appeared disturbed in body regions next to strong accumulation of fluid, whereas in regions where less tissue swelling occurred it was stage specifically differentiated, e.g. in caudal regions (supplementary material Fig. S1).

lacZ reporter gene expression during embryogenesis

Cx26 promoter activity, identified by β-gal staining, was not detected before E10.5. Between E10.5 and 17.5, Cx26+/floxed/lacZ[Cx32] embryos showed increasing lacZ expression in the developing skin of the embryo, in subcutaneous jugular regions, in the developing subarachnoidal space (Fig. 5A,B) and in the placenta (supplementary material Fig. S3).

The earliest β-gal activity was found on E10.5 in dorsolateral parts of the embryonic mesenchyme and in the regions of the peripheral lymphatic capillary network of Cx26+/Cx32 embryos. Therefore we analyzed the whole developing lymphatic vascular system of Cx26+/Cx32 embryos using lymphatic endothelial-specific marker proteins. Expression of LYVE-1, the earliest known marker of lymphatic endothelium (Tammela and Alitalo, 2010), was investigated in whole-mount embryos and embryo sections. In comparison with Cx26+/+ or Cx26+/floxed/lacZ[Cx32] embryos the peripheral lymphatic capillary network of Cx26+/Cx32 embryos was strongly reduced or even absent on E14.5 (Fig. 4A–D). At the same stage dermal blood vasculature appeared normal. The connecting network of dermal lymphatic capillaries was missing, and from E14.5 onwards the embryos developed lymphedemas. By contrast, Cx26+/+Cx32 embryos showed normally developed early lymphatic anlagen, i.e. jugular lymph sacs, when compared with control Cx26+/floxed/lacZ[Cx32] embryos (Fig. 4E,F). Immunofluorescence analyses of the lymphatic endothelial-specific marker protein Prox1 on E15.5 also demonstrated the lack of dermal lymphatic vessels in Cx26+/Cx32 embryos, while in the deep embryo, Prox1-positive lymphatics were present (Fig. 4G–J). These findings suggest that the permutation affects development of peripheral lymphatics but not deeper parts of the lymphatic vessel system. Taken together, lethality of Cx26+/Cx32 embryos was not caused by a defective placental function, skin development or blood vessel morphology but by severe lymphedemas, which were the consequence of an impaired dermal lymphatic capillary network.

Fig. 2. Histological analyses of embryonic lethal Cx26+/Cx32 embryos. (A,B) Comparison of Cx26+/Cx32 embryos and control littermates (Cx26+/+). Cx26+/Cx32 embryos die between E16.5 and 18.5 showing accumulations of tissue fluid (lymphedemas; asterisk) and agnathia. Malformation of Cx26+/Cx32 embryos only become macroscopically visible after E14.5. (C–F) Paraaffin sagittal sections of (C,E) control embryo (Cx26+/+) and (D,F) Cx26+/Cx32 littermate on E16.5, showing severe swelling of the subcutaneous mesenchyme (asterisk). Most organs seem to be normally developed, but the embryo exhibits severe lymphedemas. Scale bars: 1 mm (A–D); 250 μm (E,F).
branchial arches (Fig. 5A). From E10.5 to 13.5 it was present in the ectoderm in a highly regional manner, emerging more laterally than ventrally and dorsally in the embryonic body. Later, between E14.5 and 17.5, β-gal activity appeared more uniformly distributed in the epidermis (Fig. 5A). In addition to being present in embryonic skin, reporter gene expression was strongly abundant in the back of the Cx26+/Cx32 embryo (the arrow indicates a blood vessel). (D-G) Whole-mount immunohistochemical staining (E14.5) for the blood endothelial marker MECA-32. No differences in the dermal blood vessel network are evident in a Cx26−/−;Cx32 embryo and a control littermate (Cx26+/−). Scale bars: 1 mm (A,B); 500 μm (C); 250 μm (D,E); 125 μm (F,G).

Expression level of Cx26 and transgenic Cx32
Because it was known for other organs that the ablation of one connexin isoform can influence the level of another connexin isoform (Simon and McWhorter, 2003), we investigated whether the monoallelic expression of ectopic Cx32 influenced expression of Cx26.

Therefore we determined the amount of Cx26 in embryonic skin (Fig. 6A,B) and in placenta (Fig. 6C–E). In Cx26−/−;lacZ[Cx32] embryonic skin, the expression of the unaltered Cx26 allele was sufficient for the production of the normal level of Cx26, which was similar to that in Cx26+/+ controls. But Cx26 protein was significantly reduced in Cx26−/−;Cx32 embryonic skin compared with Cx26+/−/lacZ[Cx32] skin, although both genotypes were heterozygous for Cx26 (Fig. 6A,B). Because of the very low amount of Cx26 protein relative to the total protein content in embryonic skin lysates, we focused on Cx26 protein ratios and transcripts in the placenta. We chose the placenta, which in the mouse is of exclusive embryonic origin, because reporter gene analysis showed very strong expression in the labyrinth (cf. supplementary material Fig. S3). In comparison with Cx26+/+ placenta, Cx26−/−;lacZ[Cx32] and Cx26−/−;lacZ[Cx32]neo placentas showed nearly a 50% reduction in Cx26 protein (Fig. 6C,D). This is in contrast to Cx26−/−;lacZ[Cx32] embryonic skin, where monoallelic expression was sufficient for normal Cx26 expression. However, the reduced Cx26 in these placentas seemed to be sufficient for normal development of the mice, because they did not show any abnormalities. Remarkably, in comparison with the genotypes Cx26−/−;lacZ[Cx32] and Cx26−/−;lacZ[Cx32]neo, placentas of the genotypes Cx26+/−/lacZ[Cx32] exhibited a further significant decrease of Cx26 protein level (Fig. 6C,D). Thus in Cx26+/−;lacZ[Cx32] embryonic skin as well as placentas, Cx26 was abnormally reduced compared with Cx26+/−/lacZ[Cx32] tissues. This finding was confirmed by a strong loss of Cx26 immunostaining and strong Cx32 immunosignals in the Cx26+/−;lacZ[Cx32] placental labyrinth (supplementary material Fig. S5). On the transcript level, the genotypes Cx26+/−/lacZ[Cx32], Cx26−/−;lacZ[Cx32]neo, Cx26+/−;Cx32 and Cx26−/−;Cx32neo expressed comparable amounts of Cx26 mRNA but this was approximately half of the level found in Cx26+/+ placenta (Fig. 6E). Thus transcript levels were proportional to protein levels in Cx26+/−/lacZ[Cx32] and Cx26−/−;lacZ[Cx32]neo placentas, whereas in Cx26−/−;lacZ[Cx32] and Cx26−/−;Cx32neo placentas the Cx26 protein levels were proportionally lower than the transcript levels. This finding indicates a posttranscriptional reduction of Cx26 in Cx26−/−;lacZ[Cx32] placentas. As expected, transgenic Cx32 mRNA was only found in the genotypes Cx26+/−;Cx32 and Cx26−/−;Cx32neo, whereas no endogenous Cx32 mRNA was expressed in placentas (Fig. 6E). In summary, ectopic expression of Cx32 led to an abnormal reduction of the Cx26 protein level in Cx26+/−;lacZ[Cx32] embryonic skin and placenta.

Cell-type-specific Cx26/KICx32
The pattern of the reporter gene expression raised the question of which Cx26-expressing cell populations are involved in malformations and embryonic lethality of heterozygous Cx26KICx32 embryos. To discriminate between diverse cell populations we used three different transgenic mouse lines that expressed Cre recombinase under different promoter elements for the generation of heterozygous, cell-type-specific Cx26KICx32 mice.

Since Cx26 expression was found in different regions of the mesenchyme, we tested whether development of lymphatic capillaries is disturbed when Cre recombinase is active in mesenchymal cells in the genetic background of Cx26KICx32 mice. Therefore we mated female Cx26−/−;lacZ[Cx32] mice with male Prxl−/− mice, in which Cre activity is directed to the mesenchyme of the limb buds, the interlimb flanks and craniofacial mesenchyme (Logan et al., 2002). The resulting Cx26+/−/lacZ[Cx32]Prxl−/− mice were viable and showed no obvious phenotypic abnormalities (data not shown). Because it has been reported that in mammals lymphatic endothelial cells originate from a subpopulation of venous endothelial cells, we investigated the effect in Cx26KICx32 mice when Cre expression is directed to blood endothelial cells. Hence we bred male Tie2−/− mice (Constien et al., 2001) with female Cx26+/−/lacZ[Cx32] mice. The
gene Tie2 codes for a receptor tyrosine kinase and its promoter is active in blood endothelial cells (Sato et al., 1993; Takakura et al., 1998). Interestingly, also the obtained Cx26+/loxZ[Cx32], Tie2–Cre mice were viable and showed no obvious phenotypic abnormalities (data not shown). The fact that Cx26+/loxZ[Cx32], Prx1–Cre and Cx26+/loxZ[Cx32], Tie2–Cre mice did not exhibit lymphatic abnormalities suggests that neither the Prx1–Cre-positive mesenchyme nor the Tie2–Cre-positive endothelium are responsible for disturbed lymphangiogenesis of Cx26KICx32 embryos.

Next, we investigated whether the monoallelic ectodermal-directed replacement of Cx26 with Cx32 contributes to the lymphatic phenotype of Cx26KICx32 embryos. For this reason we bred male keratin 5–Cre (K5–Cre) mice (Ramirez et al., 2004) with female Cx26+/loxZ[Cx32] mice, in order to generate Cx26+/loxZ[Cx32], K5–Cre embryos. Interestingly, Cx26+/loxZ[Cx32], K5–Cre embryos exhibited a similar phenotype to Cx26+/-Cx32 embryos, which was characterized by strong lymphedemas, agnathy and embryonic death (Fig. 7A). In order to determine the cell population in which K5–Cre-mediated exchange of Cx26 by Cx32 had occurred, we compared Cx26+/loxZ[Cx32], K5–Cre embryos with Cx26+/+ and Cx26+/loxZ[Cx32] littermates. In Cx26+/loxZ[Cx32], K5–Cre embryos between E12.5 and 15.5, the floxed lacZ reporter gene was deleted (Fig. 7B–D; supplementary material Fig. S6) and transgenic Cx32 was expressed only in ectodermal cells in which the K5 and Cx26 promoters were active (supplementary material Fig. S7). Cell lineage analysis of K5–Cre mice using the R26R reporter mouse strain (Soriano, 1999) also showed that K5–Cre was exclusively expressed in stratified epithelia as mentioned by Ramirez et al. (Ramirez et al., 2004). Cx32 was not expressed in either the β-gal-positive subcutaneous mesenchyme or the subarachnoidal space, indicating that these compartments are not involved in the disturbed lymphangiogenesis of the mutants (data not shown). However, transgenic Cx32 was abundantly expressed in the developing skin of Cx26+/loxZ[Cx32], K5–Cre embryos, but not in the skin of control Cx26+/loxZ[Cx32] embryos (supplementary material Fig. S7). Our results demonstrated that between E12.5 and 15.5 the Cx26 and K5 promoters were active at the same time, in the same ectodermal cells. Although the monoallelic replacement of Cx26 with Cx32 did not take place in the entire Cx26+/loxZ[Cx32], K5–Cre embryonic epidermis, the embryos developed severe lymphedemas. Because Cx26+/loxZ[Cx32], K5–Cre embryos also exhibited the lymphatic defects observed in Cx26KICx32 embryos, we investigated whether dermal lymphatics were also absent. Whole-mount immunohistochemical staining for LYVE-1 demonstrated a strong reduction of the connecting lymphatic vasculature network in Cx26+/loxZ[Cx32], K5–Cre embryos (Fig. 7E–H), similar to the ubiquitous Cx26KICx32 embryos.

These findings confirm that Cx26 in ectodermal cells is involved in peripheral lymphangiogenesis. Heterozygous replacement of Cx26 with Cx32, when restricted to ectodermal cells (mediated by K5–Cre), leads to malformations and strong decrease of the peripheral lymphatic network in Cx26+/loxZ[Cx32], K5–Cre embryos, producing severe lymphedemas.

Expression analyses of growth factors and receptors relevant for lymphangiogenesis

To examine whether relevant factors for lymphangiogenesis were altered in Cx26+/loxZ[Cx32], K5–Cre embryonic skin, we tested the
expression of several factors on E14.5 by quantitative real-time polymerase chain reaction (qRT-PCR) analyses. We investigated the transcript levels of VEGF-C, VEGF-D, the corresponding receptors VEGFR-2, VEGFR-3 and the soluble form of VEGFR-2 (sVEGFR-2), which is known to antagonize VEGF-C action (Albuquerque et al., 2009). Furthermore, we tested neuropilin-2, a transmembrane receptor, whose interaction with neuropilin-2, a transmembrane receptor, whose interaction with neuropilin-2, a transmembrane receptor, whose interaction with VEGFR-2 (sVEGFR-2), which is known to antagonize VEGF-C action (Albuquerque et al., 2009). Furthermore, we tested neuropilin-2, a transmembrane receptor, whose interaction with neuropilin-2, a transmembrane receptor, whose interaction with neuropilin-2, a transmembrane receptor, whose interaction with VEGFR-2 (sVEGFR-2), which is known to antagonize VEGF-C action (Albuquerque et al., 2009). 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Ectodermal Cx26 function is essential for peripheral lymphangiogenesis

To determine whether the loss of Cx26 function, the gain of ectopic Cx32 function or both are responsible for the lymphatic phenotype of Cx26+/loxP[Cx32]K5Cre embryos, we examined mice in which Cx26 was deleted in ectoderm (loss of function). Therefore, we bred male Cx26−/−:K5Cre mice with female Cx26+/+ mice (Cohen-Salmon et al., 2002), in which the coding region of Cx26 was flanked by loxp sites (floxed). We obtained no living offspring of the genotype Cx26+/Cx32K5Cre, because embryos developed lymphedemas and died between E16 and 18.5 (Fig. 9A,B). In comparison with Cx26+/loxP[Cx32]K5Cre embryos, which developed severe lymphedemas, Cx26+/loxP[Cx32]K5Cre embryos exhibited a milder lymphatic phenotype. The extent of lymph accumulation in Cx26+/loxP[Cx32]K5Cre embryos was in some cases severe, but appeared milder in others. In accordance with this, the lymphatic capillary meshwork of Cx26+/loxP[Cx32]K5Cre embryos
had been established but was much less interconnected compared with controls (Fig. 9C–F). In detail, the dermal lymphatic system of Cx26^fl/fl :K5–Cre embryos appeared coarsely netted with enlarged diameter vessels, but the fine vessels were missing (Fig. 9E,F). The less severe phenotype is in line with the finding that K5–Cre-mediated deletion of ectodermal Cx26 between E12.5 and 15.5 does not occur in the entire ectoderm (cf. Fig. 7B–D; supplementary material Fig. S6). During embryogenesis K5–Cre activity leads to a mosaic deletion of Cx26 in the ectoderm. We conclude that a partial deletion of Cx26 in the ectoderm (under control of the K5 promoter) leads to malformation of peripheral lymphatic vessels, which results in lymphedema and embryonic death in utero.

Discussion

Our study provides the first demonstration that expression of Cx26 in ectodermal cells has an impact on the formation of a functional dermal lymphatic capillary system in mice. Both, ablation of ectodermal Cx26 or replacement of ectodermal Cx26 with Cx32 affect peripheral lymphangiogenesis and cause severe lymphedemas that result in embryonic death.

Because the fluid balance in Cx26^+/Cx32 mutant embryos was altered after E14.5, we investigated the developing vascular systems. We found no evidence for cardiac or blood vascular defects. Therefore, we focused on embryonic lymphangiogenesis, which starts relatively late, i.e. subsequently to cardiovascular development. Although, the very first anlagen of the lymphatic vascular system, the jugular lymph sacs and also deep lymphatics, appeared normally developed in Cx26^+/Cx32, Cx26^+/

\[\text{Cx26}^\text{fl} : \text{K5–Cre} \] embryos, the dermal lymphatic capillary system was nearly absent on E14.5. The reduction of peripheral dermal lymphatics in Cx26^+/Cx32, Cx26^+/\text{K5–Cre} and Cx26^0/0:K5–Cre embryos was detected at a developmental stage when the blood vascular system appeared normal. Thus we conclude that ablation or replacement of Cx26 with Cx32 in ectoderm specifically affected peripheral lymphangiogenesis and cause severe lymphedemas that result in embryonic death.

Because the skin appeared thinner in Cx26^+/Cx32 embryos on E16.5, we cannot exclude functional abnormalities caused by the lack of Cx26 or the presence of Cx32. However, the epidermis appeared defective only in dorsal regions of later staged embryos.
Fig. 9. Peripheral lymphangiogenesis depends on ectodermal Cx26. (A,B) Cx26fl/fl;K5–Cre embryo in comparison with control littermate (Cx26+/+) on E17.5. Cx26fl/fl;K5–Cre embryos are non viable and develop lymphedemias similar to Cx26+/– and Cx26fl/fl;K5–Cre embryos. (C–F) Whole-mount immunohistochemical staining of LYVE-1 to show the lymphatic vasculature in Cx26fl/fl;K5–Cre and control (Cx26+/+) embryos on E14.5. In comparison with the control littermate, the Cx26fl/fl;K5–Cre embryo lacks the fine dermal lymphatic vessels and shows coarsely connected dermal lymphatic capillaries. Scale bars: 1 mm (A–D); 250 μm (E,F).

Analyses of the mouse mutants Cx26+/loxlacZ[Cx32];Pral1–Cre and Cx26+/loxlacZ[Cx32];Tie2–Cre showed that neither the mesenchyme nor the blood endothelium are responsible for the lymphatic abnormalities in Cx26KICx32 mice. Instead, K5–Cre-mediated replacement of Cx26 with Cx32 (Cx26+/loxlacZ[Cx32];K5–Cre embryos) or deletion of both Cx26 alleles resulted in similar phenotypic abnormalities as found in Cx26–/–/Cx32 embryos. Cx26+/loxlacZ[Cx32];K5–Cre embryos, in which the replacement of Cx26 with Cx32 is restricted to the ectoderm, are embryonic lethal as a result of the lack of a peripheral lymphatic network. K5–Cre-mediated replacement of Cx26 with Cx32 exclusively occurred in ectodermal cells of the developing skin. Although the exchange did not take place in the whole ectoderm, the heterozygous replacement of Cx26 with Cx32 led to strong phenotypic abnormalities.

In case of Cx26+/–/Cx32 embryos, the expression of Cx26 protein was severely reduced when Cx32 protein was expressed ectopically. Previously, we had found that Cx26 protein was reduced in Cx32-deficient mice, although Cx26 mRNA was not affected (Nelles et al., 1996). However, consistent with the results of Nelles et al., no alteration in the level of Cx26 mRNA could be detected in Cx26–/–/Cx32 embryonic tissue. These findings indicate a transdominant effect of ectopic Cx32 expression on Cx26 expression, suggesting a posttranscriptional mechanism.

Loss of ectodermal Cx26 in Cx26+/–;K5–Cre embryos also induced lymphedemias and embryonic death as a result of reduction of peripheral lymphatics. These findings demonstrate an essential role of ectodermal Cx26, because loss of Cx26 function produced malformed dermal lymphatics, which were not capable of maintaining tissue fluid homeostasis. But, because the lymphatic phenotype of some Cx26+/–;K5–Cre embryos was milder than in Cx26+/–/Cx32 or Cx26+/loxlacZ[Cx32];K5–Cre embryos, but still embryonically lethal, it is possible that ectopic Cx32 expression aggravates the phenotypic abnormalities caused by reduction of Cx26. Stoichiometry of Cx26, Cx32 and heteromeric Cx26–Cx32 channels varies and enables cells to dynamically regulate their intercellular communication (Ayad et al., 2006). For instance heterotypic Cx26–Cx32 channels are selectively permeable to second messengers, such as cyclic nucleotides and inositol phosphates, whereas homomeric Cx26 or Cx32 channels are not (Ayad et al., 2006; Locke et al., 2004). We showed that the Cx26 protein level was significantly reduced in Cx26+/–/Cx32 embryos, but the remaining Cx26 still co-localized with ectopic Cx32. Probably, heteromeric Cx26–Cx32 or ectopic Cx32 channel properties additionally perturb Cx26-mediated intercellular signaling, which is needed for development of peripheral lymphatics.

Interestingly, in contrast to the ectoderm it is probable that in the placenta Cx32 can replace Cx26. It has been shown that deletion of Cx26 in the placenta leads to embryonic death on E10.5 (Gabriel et al., 1998). However, the observed reduction of Cx26 (~90%) in the placental labyrinth seems to be compensated by ectopic expression of Cx32, because these embryos are not growth retarded. Moreover this was shown with Cx26+/loxlacZ[Cx32];K5–Cre and Cx26+/loxlacZ[Cx32];K5–Cre embryos, which exhibited similar phenotypic abnormalities as Cx26+/–/Cx32 embryos, although K5–Cre is not active in the placental labyrinth. These findings indicate that Cx32 can replace Cx26 in the placenta.

Our results suggest an essential role of Cx26-expressing ectodermal cells that can influence peripheral lymphangiogenesis, potentially by regulating lymphangiogenic signals from the early epidermis to the developing dermal lymphatic endothelium.
Incidentally, it has been shown in humans that Cx26 expression is associated with lymphatic vessel invasion, large tumor size and poor prognosis in human breast cancer (Naoi et al., 2007). The authors conclude that Cx26 seems to enhance metastasis, probably by promoting invasion of the lymphatic vessels. Very recently, mutations in GJC2 (Cx47), another member of the connexin family, have been reported to cause primary lymphedema in humans (Ferrell et al., 2010; Ostergaard et al., 2011). With this in mind, it appears promising to further analyze connexin protein expression and function during development and maintenance of the lymphatic vessel system in mice and humans.

**Materials and Methods**

All mice used in this study were kept under standard housing conditions with a 12-hour:12-hour dark-light cycle and with food and water ad libitum. All experiments were carried out in accordance with local and state regulations for research with animals.

**Cloning of the conditional Cx26lacZKClαc32 targeting vector and ES cell transfection**

The 1.9 kb 5′and the 5.0 kb 3′ homologous regions were PCR amplified and cloned in pBluescript (Stratagene). A PCR fragment (106 bp) including 87 bp of the Cx26 (G/J2) splice acceptor site and 6 bp of exon2 of Cx26 was cloned with SmaI in pBluescript and used twice in the final targeting vector, once upstream of the nls-lacZ and the second time upstream of the coding region of Cx32 (G/J2). The Cx26 splice acceptor nls-lacZ reporter gene fragment, being a SV40 poly(A) signal, was blunt-end cloned (SmaI–Sall; Klenow) between the two IoxP sites of the vector pHW01, which had been EcoRI digested and blunted. The Cx32 coding region (489 bp) was amplified by PCR (894 bp) introducing a NiR restriction site 3′ of the stop codon and this fragment was cloned downstream of the Cx26 splice acceptor sequence. The introduced NiR site was used to incorporate a neomycin selection cassette flanked by IoxP sites. The final construct was transformed in bacteria constitutively expressing Fip recombine or Cre recombinase, to test for the deletion of the fl-=loxP-flanked regions, and was finally partially sequenced by the AGOWA company (Berlin, Germany) (Theis et al., 2000).

NiR-linearized targeting vector DNA (333 μg) was used to transfect HM1 (Magin et al., 1992) embryonic stem (ES) cells. Selection, PCR screening, Southern blot analysis (DNA digested with EcoRI).

**Generation and genotyping of Cx26-lacZfloxed/Cx32hofigo/Cx26-lacZfloxed/Cx32hofigo**

By transfection with the targeting vector, one out of 680 ES cell clones had undergone homologous recombination and was used for blastocyst injection. After ES-cell transfection and embryonic resorption of the recombinated allele, Cx26-lacZfloxed/Cx32hofigo mice were obtained. These mice were bred with deleter-flip mice (Rodriguez et al., 2000) to obtain Cx26-lacZfloxed/Cx32hofigo mice, in which the IoxP-flanked selection cassette was removed. In order to obtain the embryonic-lentil genotypes Cx26-lacZfloxed/Cx32hofigo or Cx26-3′, ubiquitous Cre-recombinase-expressing mice (POK-Cre) (Lallemand et al., 1998) were mated with Cx26-lacZfloxed/Cx32hofigo mice and the embryos were examined at various stages after timed matings. For cell-type-specific deletion of the IoxP-flanked lacZ reporter gene, we bred female Cx26-lacZfloxed/Cx32hofigo mice with male Tie2-Cre mice (Consten et al., 2001), male keratin 5 (K5)-Cre mice (Ramirez et al., 2004) or male Prxl–Cre mice (Logan et al., 2002). Cx26-mutated mice and embryos were genotyped (using tail DNA and yolk sac or tail DNA, respectively) by PCR using the primers Cx26 forward (5′–AGC AGC AGC GTA GAG GAG GCC GTT TGT TTG G3′), Cx26 reverse (5′–GGA CCC TTC TAC AGC GAG CTT CTG GGG 3′), lacZ reverse (5′–CCA TTC AGC CGG AAC TGT TGG-3′) and Cx32 reverse (5′–GCT CTC AGC AGC AAC CAC CAG CAC-3′).

**Southern blot hybridization**

Embryos (E15.5) of resulting transgenic genotypes were tested by Southern blot hybridization. For this purpose, isolated DNA of each genotype was digested with BglII. After electrophoretic separation on agarose gels, blotting onto Hybond N membranes (Amersham Biosciences) and ultraviolet-meridional crossinglinking for fixation, hybridization was performed under stringent conditions using the QuickHyb solution (Stratagene) at 68°C for 1.5 hours. An ~1 kb BglII fragment of the 5′ homology region of Cx26 was used as the external probe and a 0.5 kb Neol–Xbal fragment of the Cx32 coding region served as the internal probe.

**Northern blot hybridization**

Total RNA from placentas (E12.5) was collected using TRIzol™ (Invitrogen) according to the manufacturer’s protocol. Loaded aliquots were electrophoretically separated, blotted and hybridized as described previously (Hennemann et al., 1992). For detection of Cx26 mRNA, a 460 bp BglII–BglII fragment of the Cx26 coding region, and for Cx32 mRNA, a 450 bp EcoNI fragment of the Cx32 coding region were used for generation of the probes.

**RNA preparation and cDNA synthesis**

The skin of Cx26-flox/Cx32hofigo K5-Cre embryos (n=4) and Cx26-3′ and Cx26-3′/loxP/Cx32hofigo control littersmaters (n=3) was dissected and pooled on E14.5. Total RNA from embryonic skin was collected using TRIzol™ (Invitrogen) according to the manufacturer’s protocol. Reverse transcription of 2 μg RNA included a DNase I (Invitrogen) treatment and was performed using Oligo(dT)16 primer (Invitrogen) and Moloney murine leukemia virus reverse transcriptase (Invitrogen) according to the protocol supplied.

**Quantitative PCR**

Quantitative real-time PCR was performed in triplicate using an ABI Prism 7300 Sequence Detector (Applied Biosystems). For qRT-PCR, 1 μl of first strand cDNA solution was diluted in a final volume of 20 μl containing 0.2 pmol of each gene-specific primer and 10 μl Power SYBR® Green PCR Master Mix (Applied Biosystems) including a ROX™ dye as an internal passive reference. The signal intensities of VEGF-C, VEGF-D, VEGFR-2, sVEGFR-2, VEGF-3 and neuropilin-2 transcripts were quantified using real dilution series of gene-specific standard cDNA. β-Actin cDNA was used as a standard for normalization. Subsequent melting curve analysis was used to determine the specificity of the PCR fragments. A tenfold dilution series of purified PCR products ranging from 1 pg to 0.1 fg were used to create a standard curve for each gene. The PCR reactions were carried out in triplicate. All primer sequences are shown in supplementary Table S2.

**Immunoblot analysis**

Embryos or placenta were solubilized in RIPA buffer or 1× Complete buffer (Roche) and sonicated three times for 10 seconds while being incubated on ice. Immunoblotting was performed as described by Dobrowski et al. et al. using mouse anti-Cx26 (1:750; Zymed) and goat anti-mouse horseradish-peroxidase (HRP)-conjugated antibodies (1:10,000; Dianova, Hamburg, Germany) (Dobrowski et al., 2008). The concentration of the loaded proteins was analyzed by immunoblotting using anti-β-actin antibodies (1:500; Sigma). Protein expression was quantified using the Hochlab E.A.S.Y.Win32 quantification software (Hochlab GmbH, Wiesloch, Germany).

**Histological analysis of embryos**

Embryos were fixed in 4% paraformaldehyde (PFA) or Bouin solution, dehydrated in a graded series of ethanol, embedded in paraffin plus (Sherwood Medical Industries, St Louis, MO). Paraffin serial sections (4–8 μm) were stained with Hematoxylin and acid fuchsin or Hematoxylin and Eosin.

**Whole-mount staining of embryos for β-gal**

After dissection, E10.5–14.5 embryos were fixed for 30–120 minutes in 4% PFA or 0.2% glutaraldehyde or both at 4°C. Staining for β-gal was performed as previously described (Krüger et al., 2000).

**Staining of cryosections for β-gal followed by immunohistochemical analysis**

Cryostat sections (10–25 μm) were fixed with 0.2% glutaraldehyde for 5 minutes, or for combined β-gal staining and immunohistochemical analysis on the same section, in 2% PFA and 0.1% glutaraldehyde for 2 minutes. After washing steps, staining for β-gal was performed using the substrate 5-bromo-4-chloro-3-indoly-β-galactoside (X-gal). After X-gal staining, sections were post-fixed for 5 minutes in 4% PFA and immunohistochemical staining was carried out using the M.O.M.™ Immunodetection Kit (Vector Laboratories) following the manufacturer’s protocol. Sections were incubated with rabbit anti-LYVE-1 IgG (1:200; Reliatech, Braunschweig, Germany). Secondary antibody incubation was then carried out with biotinylated goat anti-rabbit IgG (1.500; Zymed) for 45 minutes at room temperature. The immunocomplexes formed were visualized by peroxidase enzyme reaction containing 3.3′-diaminobenzidine tetrahydrochloride (Sigma-Aldrich, Steinheim, Germany) and <3 mmol/l H2O2 for ~10 min. After staining, sections were rapidly washed, counter-stained with 0.1% Eosin and mounted in Entellan (Merck, Hohenbrunn, Germany).

**Immunofluorescence analysis**

Embryos and placentas were frozen on dry ice at –70°C and embedded in TissueTec (Miles Inc., Elkhart, USA). Cryostat sections (12–25 μm) were fixed for 5 minutes with absolute ethanol at –20°C or with 4% PFA; washed three times with PBS without calcium and magnesium (PBS) and blocked in 4% normal goat serum or 4% bovine serum albumin, 0.1% Triton X-100 in PBS for 1 hour. The following primary antibodies were used: rabbit anti-Cx26 (1:400–1:800; Zymed), mouse anti- Cx32 (1:300–1:600; Zymed) and rabbit anti-Prox1 (1:100; Reliatech) in blocking solution. After incubation overnight, the sections were washed three times in PBS and afterwards incubated for 1 hour at room temperature with secondary antibodies: goat anti-rabbit conjugated to Alexa Fluor 594 (1:1000) and goat anti-mouse conjugated to Alexa Fluor 488 (1:1000; Mobitech, Gottingen, Germany). Nuclei
were stained with Hoechst 33258 (1:20,000; Sigma-Aldrich). After additional PBS washing steps, sections were embedded in Dako Glycergel mounting medium (Dako, Hamburg, Germany) and analyzed by confocal microscopy (LSM 510 and LSM 710, Zeiss).

Whole-mount immunohistochemical analysis
Immunohistochemical staining of whole-mount embryos was performed as described previously (Nagy et al., 2003) but modified by using the M.O.M.™ ImmunoDetection Kit (Vector Laboratories) following the manufacturer’s protocol. As primary antibodies, rabbit anti-LYVE-1 IgG and rat anti-MECA-32 IgG (1:50, BD Pharmingen, Franklin Lakes, USA) were used.

We thank Gerda Hertig and Christine Siegmund for excellent technical assistance. This work is part of the PhD thesis of N.D. who was generously supported by a stipend from the Jürgen-Manchot Foundation (Düsseldorf). The Cx26+/-loxlacZ/Cx32neo mice were generated during the PhD work of N.P. In addition, K.W. acknowledges support of the German Research Foundation (SFB 645, B2) and Bonn University.

Supplementary material available online at http://jcs.biologists.org/cgi/content/full/124/6/2806/DC1

References


Table S1. Summary of genotypes used in this study and their specificity

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Specificity</th>
<th>Phenotype / Utilisation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cx26^{+/+}</td>
<td>Heterozygous exchange of Cx26 cDNA by floxed LacZ cDNA under control of the Cx26 promoter. Cx26 is expressed from the wild-type allele and β-galactosidase from the mutated allele. Ectopic Cx32 is not expressed.</td>
<td>No phenotype -Cx26 reporter (LacZ) is expressed</td>
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<tr>
<td>Cx26^{+/+}LacZ[Cx32]neo</td>
<td>Same as above with neomycin selection cassette</td>
<td>No phenotype</td>
</tr>
<tr>
<td>Cx26^{+/+}Cx32</td>
<td>General heterozygous exchange of Cx26 cDNA by Cx32 cDNA under control of the Cx26 promoter. LacZ is deleted and not expressed.</td>
<td>Lymphatic phenotype</td>
</tr>
<tr>
<td>Cx26^{+/+}LacZ[Cx32]neo</td>
<td>Same as above with neomycin selection cassette</td>
<td>Lymphatic phenotype</td>
</tr>
<tr>
<td>Cx26^{+/+}LacZ[Tie2-Cre]</td>
<td>Tie2-Cre mediated recombination in endothelial cells.</td>
<td>No phenotype</td>
</tr>
<tr>
<td>Cx26^{+/+}LacZ[prx1-Cre]</td>
<td>Prx1-Cre mediated recombination in mesenchymal cells</td>
<td>No phenotype</td>
</tr>
<tr>
<td>Cx26^{+/+}LacZ[K5-Cre]</td>
<td>Keratin5-Cre mediated recombination in ectodermal cells. Cells in which the Cx26 and the K5 promoter are active express Cx32 instead of Cx26 heterozygously. Cells which exhibit Cx26 promoter activity but no K5 promoter activity express LacZ heterozygously.</td>
<td>Lymphatic phenotype</td>
</tr>
<tr>
<td>Cx26^{fl/fl}</td>
<td>Cx26 coding region is floxed</td>
<td>No phenotype</td>
</tr>
<tr>
<td>Cx26^{fl/fl}K5-Cre</td>
<td>Keratin5-Cre mediated deletion of Cx26 coding region in ectoderm</td>
<td>Lymphatic phenotype</td>
</tr>
<tr>
<td>R26R:K5-Cre</td>
<td>Keratin5-Cre mediated deletion of a floxed stop-cassette leads to LacZ expression</td>
<td>No phenotype -Keratin5 promoter directed cell lineage analyses</td>
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Table S2. Primer sequences used for quantitative PCR analysis

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5′→ 3′)</th>
<th>PCR product (bp)</th>
<th>GenBank accession number</th>
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<td>VEGF-C</td>
<td>sense TGTGTCAGCGTAGATGAGC antisense TCCCTGCTCTGGTATTGAG</td>
<td>117</td>
<td>NM_009506.2</td>
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<tr>
<td>VEGF-D</td>
<td>sense GTGCAAGTAGAAAAGCCAGCC antisense CAAAGTTCCTCTGGCTGTA</td>
<td>88</td>
<td>NM_010216.1</td>
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<tr>
<td>VEGFR-2</td>
<td>sense ACCATTGAAGTGACTTGCCC antisense CCGGTTCCCATCTCTCAGTA</td>
<td>114</td>
<td>NM_010612</td>
</tr>
<tr>
<td>VEGFR-3</td>
<td>sense ACGCTGATGATAGTCCACCC antisense CTTCATCTGGGAAGAGCCTG</td>
<td>122</td>
<td>NM_008029</td>
</tr>
<tr>
<td>sVEGFR-2</td>
<td>sense CACCAGTTTGCAGAAACCTGGATGCT antisense CAATTCTGCACCCAGGGATGC</td>
<td>319</td>
<td>EU884114</td>
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<tr>
<td>Nrp2</td>
<td>sense TCAGGTAGACCTGGGGACAC antisense TCCCCAGTCCTGGCATTTAG</td>
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<tr>
<td>β-Actin</td>
<td>sense ACCAACTGGGAGCAGATGGAGAAA antisense TACGGCCAGAGGCGTACAGGGTAG</td>
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