

Nkx2.5 and Nkx2.6, Homologs of *Drosophila tinman*, Are Required for Development of the Pharynx

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Nkx2.5 and Nkx2.6 are murine homologs of *Drosophila tinman*. Their genes are expressed in the ventral region of the pharynx at early stages of embryogenesis. However, no abnormalities in the pharynges of embryos with mutations in either Nkx2.5 or Nkx2.6 have been reported. To examine the function of Nkx2.5 and Nkx2.6 in the formation of the pharynx, we generated and analyzed Nkx2.5 and Nkx2.6 double-mutant mice. Interestingly, in the double-mutant embryos, the pharynx did not form properly. Pharyngeal endodermal cells were largely missing, and the mutant pharynx was markedly dilated. Moreover, we observed enhanced apoptosis and reduced proliferation in pharyngeal endodermal cells of the double-mutant embryos. These results demonstrated a critical role of the NK-2 homeobox genes in the differentiation, proliferation, and survival of pharyngeal endodermal cells. Furthermore, the development of the atrium was less advanced in the double-mutant embryos, indicating that these two genes are essential for both pharyngeal and cardiac development.

The Nkx2.5 and Nkx2.6 genes are members of the NK-2 homeobox gene family and are closely related to the *Drosophila tinman* gene (2, 5, 21). At early stages of embryogenesis, Nkx2.5 is expressed in myocardium and pharyngeal endoderm (6, 7), while Nkx2.6 expression can be observed in pharyngeal endoderm, sinus venosa, and myocardium of the outflow tract (1, 12). In the pharynx, both Nkx2.5 and Nkx2.6 are expressed in the ventral region between embryonic day 8 (E8.0) and E8.5 (1, 7). However, by E9.0, Nkx2.6 expression becomes restricted to the lateral side of the pharynx, the pharyngeal pouches, while Nkx2.5 is still expressed on the ventral side of the pharynx, the pharyngeal floor (1, 7, 22). In the heart, redundant expression of Nkx2.5 and Nkx2.6 has been observed in the sinus venosa at E8.5 and in the outflow tract at E9.5 (1, 7). Inactivation of Nkx2.5 arrested heart formation at the looping stage, revealing a critical role of this gene at early stages of cardiac development (9, 20). However, no abnormalities in the pharynges of Nkx2.5^{-/-} embryos were reported (9, 20). Moreover, targeted disruption of Nkx2.6 did not cause any abnormalities either in the pharynx or in the heart (22). However, interestingly, in Nkx2.6^{-/-} embryos, expression of Nkx2.5 extended to the lateral side of the pharynx, suggesting a compensatory function of Nkx2.5 in the forming pharynx (22).

To investigate the function of Nkx2.5 and Nkx2.6 in the formation of the pharynx, we generated and analyzed Nkx2.5 and Nkx2.6 double-mutant mice. Interestingly, in the double-mutant embryos, the numbers of pharyngeal endodermal cells were severely reduced and the pharynges were markedly dilated, suggesting critical roles of Nkx2.5 and Nkx2.6 in pharyngeal development.

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MATERIALS AND METHODS

Generation of Nkx2.5^{-/-} Nkx2.6^{-/-} mice. The generation and viability of Nkx2.5 and Nkx2.6 knockout mice have been described previously (20, 22). The bacterial LacZ gene was inserted into the Nkx2.5 locus (20). Homozygous mutations of Nkx2.5 (Nkx2.5^{-/-} mice) were embryonically lethal, while heterozygous Nkx2.5 mutant mice (Nkx2.5^{+/-} mice) and homozygous Nkx2.6 mutant mice (Nkx2.6^{-/-} mice) were viable and fertile (20, 22). We first interbred Nkx2.5^{+/-} mice and Nkx2.6^{-/-} mice to generate Nkx2.5^{+/-} Nkx2.6^{+/-} mice, and these in turn were crossed to generate Nkx2.5^{+/-} Nkx2.6^{-/-} mice. Nkx2.5^{+/-} Nkx2.6^{-/-} mice were viable and fertile. Nkx2.5^{+/-} Nkx2.6^{-/-} mice were then crossed, and the offspring were genotyped by PCR as previously described (20). All the mutant mice were maintained in a mixed 129 and C57BL background.

Histological analysis. Mouse embryos were fixed, dehydrated, and embedded in paraffin, and in situ hybridization was performed as previously described (20). Plasmids containing full-length cDNA for HNF3 β and Shh were kindly provided by B. Hogan (Vanderbilt University Medical School, Nashville, Tenn.). The entire coding region of Pax9 was a kind gift from H. Peters (Brigham and Women's Hospital, Boston, Mass.). The plasmids for atrial natriuretic factor (ANF), B-type natriuretic peptide (BNP), myosin light chain 2A (MLC2A), and MLC2V were described previously (20). Whole-mount β -galactosidase staining was performed according to the method of Schlaeger et al. (17). TUNEL (terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling) staining and immunohistochemistry were performed as previously described (20).

RESULTS

Embryonic lethality of the Nkx2.5^{-/-} Nkx2.6^{-/-} mutation. We generated Nkx2.5^{+/-} Nkx2.6^{-/-} mice by crossing compound heterozygotes (Nkx2.5^{+/-} Nkx2.6^{+/-}; see (Materials and Methods)). Nkx2.5^{+/-} Nkx2.6^{-/-} mice were viable and fertile and exhibited no detectable abnormalities either in the pharynx or in the heart. Nkx2.5^{+/-} Nkx2.6^{-/-} mice were then intercrossed, and the offspring were genotyped. As expected, no viable Nkx2.5^{-/-} Nkx2.6^{-/-} mice were found.

Morphology and histology of Nkx2.5^{-/-} Nkx2.6^{-/-} embryos. To determine the phenotype of Nkx2.5^{-/-} Nkx2.6^{-/-} embryos, offspring of Nkx2.5^{+/-} Nkx2.6^{-/-} mice were examined during development. By E9.5, the expected Mendelian frequency of homozygous mutants was recovered (Table 1).

TABLE 1. Viability of $Nkx2.5^{-/-}$ $Nkx2.6^{-/-}$ embryos

Day of analysis	No. of embryos with phenotype		
	$Nkx2.5^{+/+}$ $Nkx2.6^{-/-}$	$Nkx2.5^{+/-}$ $Nkx2.6^{-/-}$	$Nkx2.5^{-/-}$ $Nkx2.6^{-/-}$
E8.5	14	32	16
E9.5	14	31	15
E10.5	11	19	7
E11.5	6	9	0

We first examined $Nkx2.5^{-/-}$ $Nkx2.6^{-/-}$ embryos at E9.5 and E10.5. $Nkx2.5^{-/-}$ $Nkx2.6^{-/-}$ embryos at E10.5 showed severe growth retardation and massive pericardial effusion (Fig. 1A, and B), looking similar to $Nkx2.5^{-/-}$ embryos (Fig. 1B) (9, 20). However, histological examination at E9.5 and E10.5 revealed disrupted formation of the pharynx in $Nkx2.5^{-/-}$ $Nkx2.6^{-/-}$ embryos (Fig. 1E and H). In wild-type embryos, the surface of the pharynx was covered with pharyngeal endodermal cells. A monolayer of endodermal cells covered the pharyngeal roof,

and two or three layers of cuboidal endodermal cells were observed in the pharyngeal floor and pouches (Fig. 1F). In contrast, in $Nkx2.5^{-/-}$ $Nkx2.6^{-/-}$ embryos, the number of pharyngeal endodermal cells was severely reduced and a continuous endodermal cell layer was lost. Only a small number of endodermal cells were observed, mainly on the ventral side (Fig. 1H), and the mutant pharynx showed severe dilatation (Fig. 1E and H). The abnormal dilatation was detected only in the pharyngeal part of the foregut (Fig. 1I to M). Interestingly, $Nkx2.5^{-/-}$ embryos also showed a defect in the pharynx; there were fewer endodermal cells in the pharyngeal floor and pouches, although the surface of the pharynx was completely covered by endodermal cells (Fig. 1G).

To exclude the possibility that the observed defects might be due to general growth retardation, we analyzed $Nkx2.5^{-/-}$ and $Nkx2.5^{-/-}$ $Nkx2.6^{-/-}$ embryos at earlier stages. No growth retardation was detected in $Nkx2.5^{-/-}$ or $Nkx2.5^{-/-}$ $Nkx2.6^{-/-}$ embryos by E9.0 (Fig. 2A and B). Histological analysis showed that the foregut pocket of $Nkx2.5^{-/-}$ $Nkx2.6^{-/-}$

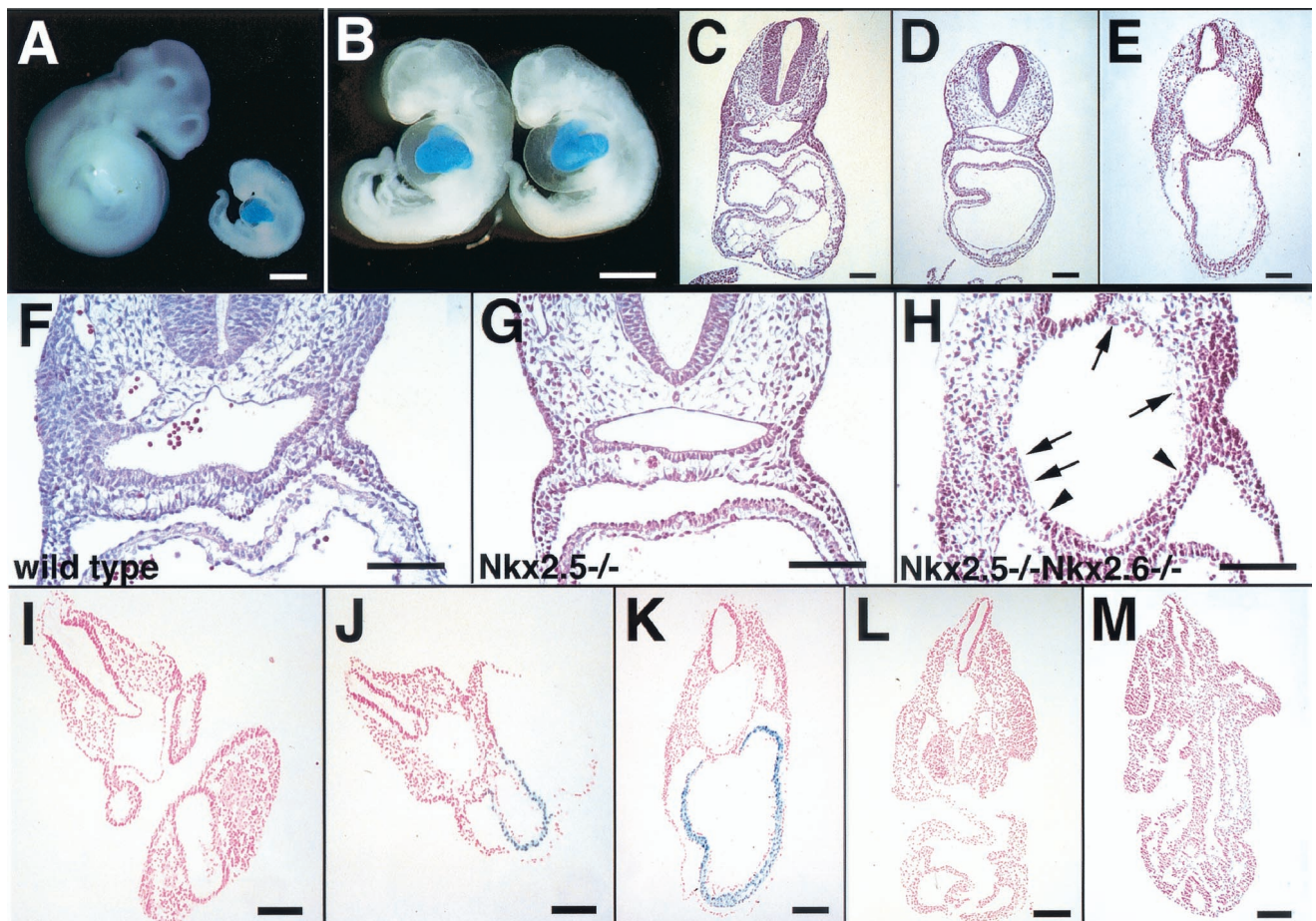


FIG. 1. Morphological and histological analysis of $Nkx2.5^{-/-}$ and $Nkx2.5^{-/-}$ $Nkx2.6^{-/-}$ embryos. (A) Whole-mount β -galactosidase staining of $Nkx2.5^{+/+}$ $Nkx2.6^{-/-}$ and $Nkx2.5^{-/-}$ $Nkx2.6^{-/-}$ embryos at E10.5 (littermates) (B) Whole-mount β -galactosidase staining of $Nkx2.5^{-/-}$ $Nkx2.6^{-/-}$ (left) and $Nkx2.5^{-/-}$ (right) embryos at E10.5. (C to E) Hematoxylin-and-eosin-stained transverse sections of wild-type (C), $Nkx2.5^{-/-}$ (D), and $Nkx2.5^{-/-}$ $Nkx2.6^{-/-}$ (E) embryos at E9.5. Note disrupted formation of the pharynxes in $Nkx2.5^{-/-}$ $Nkx2.6^{-/-}$ embryos. (F to H) Higher-magnification pictures of panels C to E. Note that a monolayer of endodermal cells remained only in a small region on the ventral side (between the arrowheads) in $Nkx2.5^{-/-}$ $Nkx2.6^{-/-}$ embryos. A small number of endodermal cells also survived in other parts of the pharynx (arrows). (I to M) Transverse sections of the double-mutant embryo shown in panel B. The disruption of pharyngeal formation was observed only in the pharyngeal part of the foregut. Bars, 200 μ m.

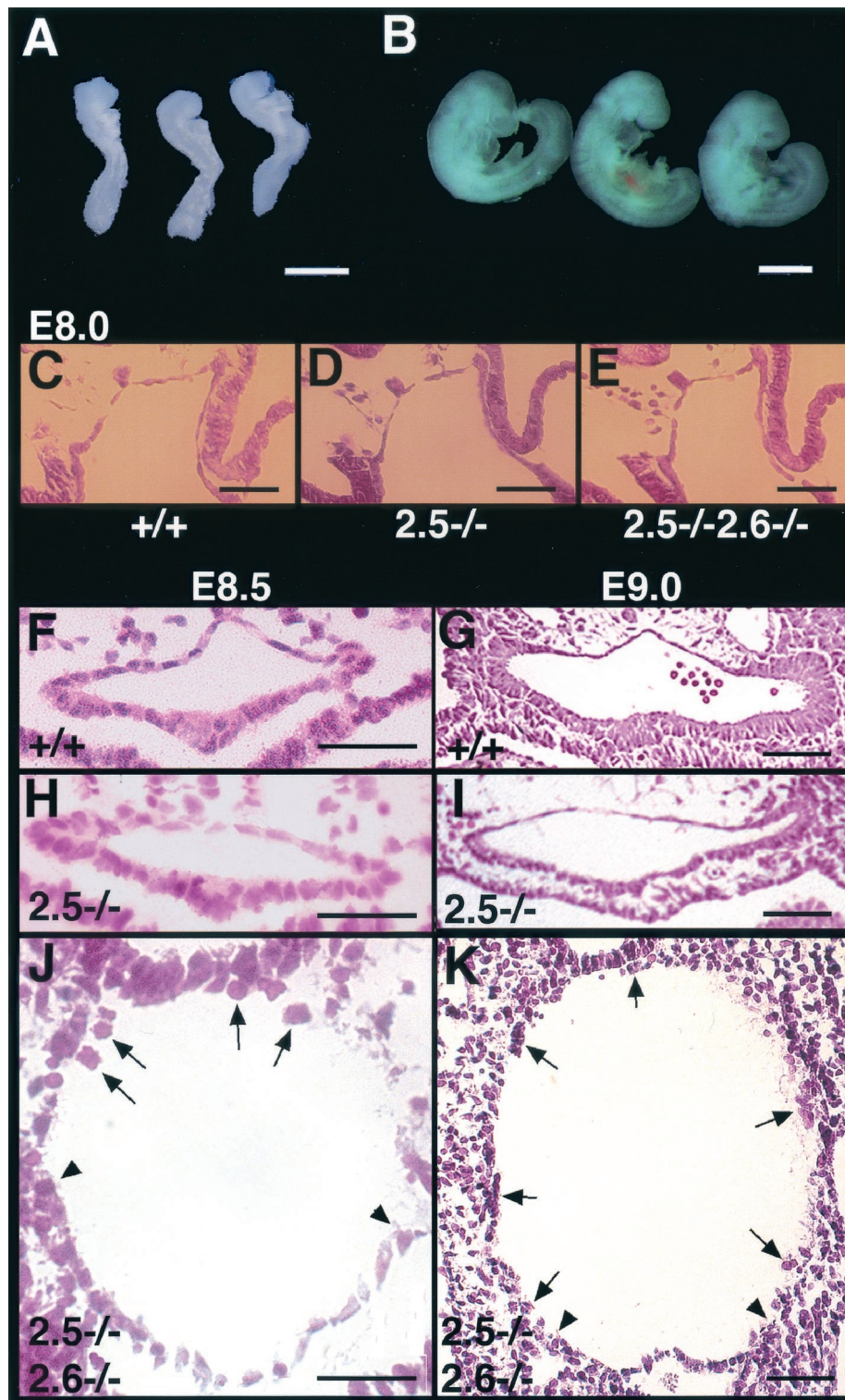


FIG. 2. (A) Wild-type (left), *Nkx2.5*^{-/-} (middle), and *Nkx2.5*^{-/-} *Nkx2.6*^{-/-} (right) embryos at E8.5, bar, 1.0 mm. (B) Wild-type (left), *Nkx2.5*^{-/-} (middle), and *Nkx2.5*^{-/-} *Nkx2.6*^{-/-} (right) embryos at E9.0. Bar, 1.0 mm. (C to E) Histology of the foregut pockets in hematoxylin- and-eosin-stained wild-type (C), *Nkx2.5*^{-/-} (D), and *Nkx2.5*^{-/-} *Nkx2.6*^{-/-} (E) embryos at E8.0. Bar, 100 μ m. (F to K) Histology of the pharynxes in wild-type (F and G), *Nkx2.5*^{-/-} (H and I), and *Nkx2.5*^{-/-} *Nkx2.6*^{-/-} (J and K) embryos at E8.5 (F, H, and J) and E9.0 (G, I, and K). (J and K) A monolayer of endodermal cells on the ventral side (between the arrowheads) and a few endodermal cells in other parts (arrows) were observed. Note that a continuous layer of endodermal cells was already lost and the pharynx was dilated in *Nkx2.5*^{-/-} *Nkx2.6*^{-/-} embryos at E8.5.

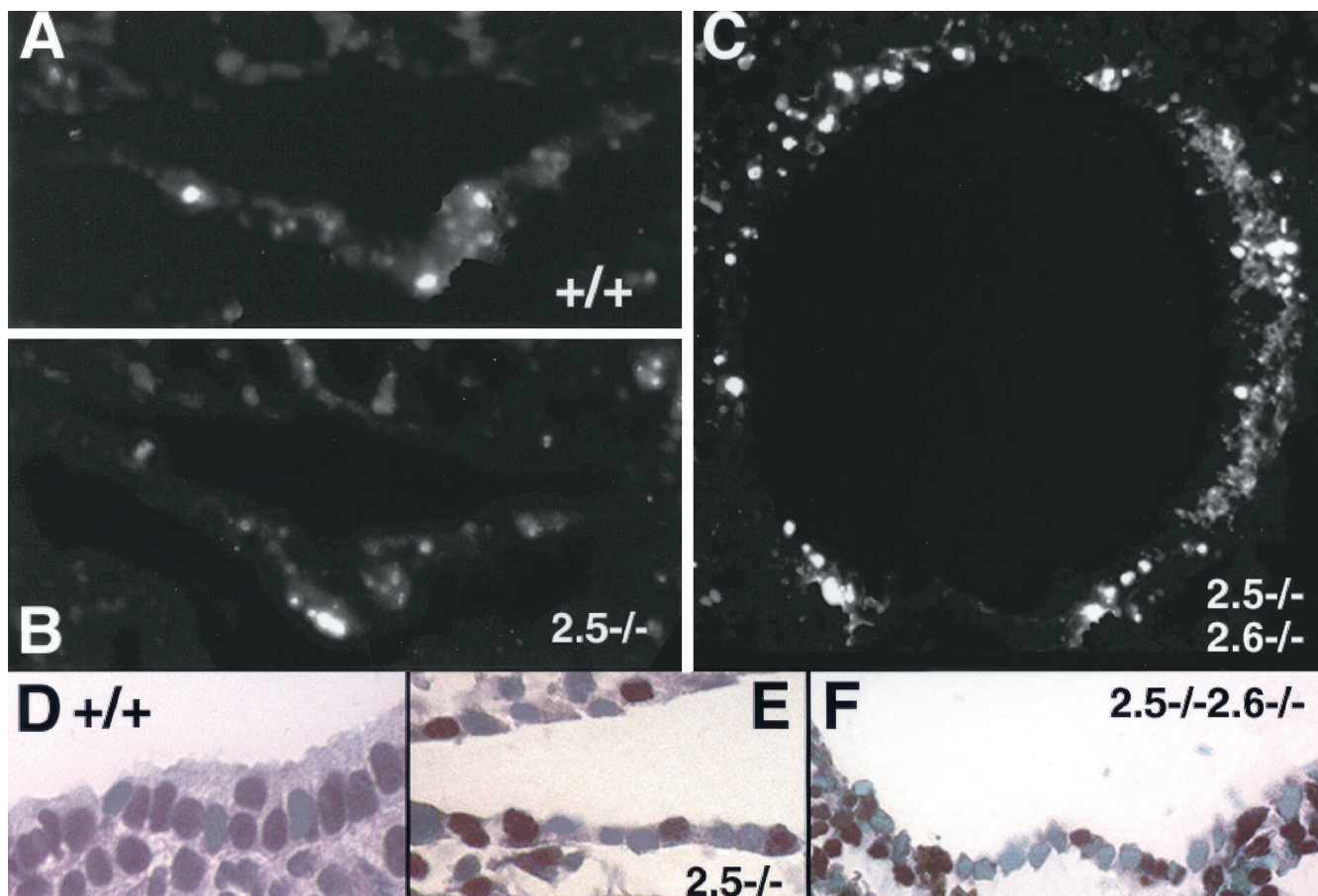


FIG. 3. TUNEL staining and PCNA staining. (A to C) TUNEL staining of transverse sections of wild-type (A), $Nkx2.5^{-/-}$ (B), and $Nkx2.5^{-/-} Nkx2.6^{-/-}$ (C) embryos at E8.75. Note enhanced apoptosis in the pharynx of $Nkx2.5^{-/-} Nkx2.6^{-/-}$ embryos. (D to F) PCNA staining of transverse sections of wild-type (D), $Nkx2.5^{-/-}$ (E), and $Nkx2.5^{-/-} Nkx2.6^{-/-}$ (F) embryos at E8.75.

embryos at E8.0 was indistinguishable from that of wild-type littermates (Fig. 2C to E). However, at E8.5, pharyngeal endodermal cells were lost in some parts of the pharynx and the pharynx was abnormally dilated in $Nkx2.5^{-/-} Nkx2.6^{-/-}$ embryos, indicating that the pharyngeal phenotype was not due to growth retardation (Fig. 2J). At this stage, no significant differences in the pharynx between $Nkx2.5^{-/-}$ and wild-type embryos were detected (Fig. 2F and H). However, at E9.0, the number of pharyngeal endodermal cells was significantly lower in $Nkx2.5^{-/-}$ embryos than in wild-type embryos, suggesting that proliferation or differentiation of these cells may be affected by inactivation of $Nkx2.5$ (Fig. 2G and L).

Enhanced apoptosis and reduced proliferation of pharyngeal endodermal cells. We then examined whether proliferation and/or apoptosis of pharyngeal endodermal cells was affected by inactivation of $Nkx2.5$ and $Nkx2.6$. TUNEL staining demonstrated that pharyngeal endodermal cells were largely apoptotic, except for a small number of cells on the ventral side in $Nkx2.5^{-/-} Nkx2.6^{-/-}$ embryos (Fig. 3C). There were no differences in the frequency of apoptotic cells in the pharynx between wild-type and $Nkx2.5^{-/-}$ embryos (Fig. 3A and B), suggesting that disruption of both $Nkx2.5$ and $Nkx2.6$ enhanced apoptosis. We next performed immunohistochemistry using anti-PCNA (proliferating cell nuclear antigen) antibody

to assess proliferation of pharyngeal endodermal cells. The rates (means \pm standard deviations) of PCNA-positive cells were 0.71 ± 0.05 , 0.43 ± 0.03 , and 0.43 ± 0.02 in wild-type, $Nkx2.5^{-/-}$, and $Nkx2.5^{-/-} Nkx2.6^{-/-}$ embryos, respectively. (Six sections from three embryos of each group were analyzed. The differences between wild-type and $Nkx2.5^{-/-}$ or $Nkx2.5^{-/-} Nkx2.6^{-/-}$ embryos were significant [$P < 0.01$].) This result indicated that proliferation of pharyngeal endodermal cells was affected by inactivation of $Nkx2.5$.

Expression of molecular markers in $Nkx2.5^{-/-} Nkx2.6^{-/-}$ embryos. To determine whether transcription of pharyngeal endoderm-specific genes was affected in $Nkx2.5^{-/-} Nkx2.6^{-/-}$ embryos, we performed in situ hybridization. Hepatocyte nuclear factor 3 β (HNF3 β) is a winged-helix transcription factor and is expressed in the node, notochord, floor plate, and embryonic endoderm (23). Expression of HNF3 β was detectable around the pharynx but was significantly down-regulated in $Nkx2.5^{-/-}$ embryos (Fig. 4A and B). Moreover, in the pharynxes of $Nkx2.5^{-/-} Nkx2.6^{-/-}$ embryos, expression of HNF3 β was lost except for endodermal cells on the ventral side and a small number of endodermal cells remaining in other parts (Fig. 4C). We next examined expression of Pax9, which is expressed in the endoderm of the pharyngeal pouches (15). In wild-type and $Nkx2.5^{-/-}$ embryos, Pax9 expression was ob-

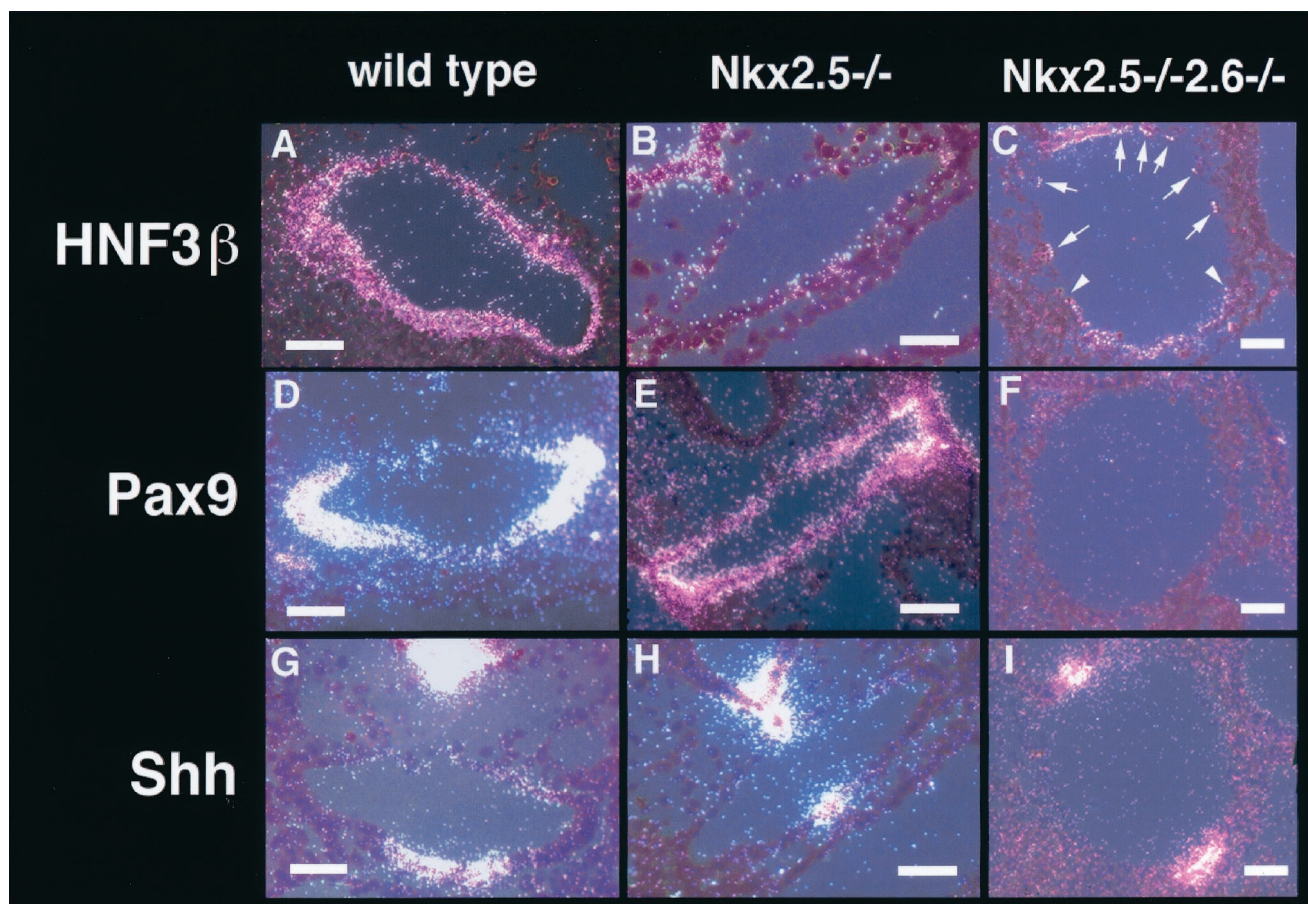


FIG. 4. Expression of pharyngeal endoderm-specific genes. In situ hybridization of transverse sections of wild-type and mutant embryos at E8.75 using probes for the indicated proteins. Bars, 100 μ m.

served in the epithelium of the pharyngeal pouches (Fig. 4D and E). However, in the pharynges of *Nkx2.5*^{-/-} *Nkx2.6*^{-/-} embryos, transcripts for Pax9 were not detected (Fig. 4F), indicating that pharyngeal pouches did not form. Sonic hedgehog (Shh) is a secreted signaling molecule and is involved in axial patterning, foregut formation, and distal limb formation (3, 8). In wild-type embryos, Shh expression was observed in the floor plate, notochord, and ventral region of the pharyngeal endoderm (Fig. 4G). In *Nkx2.5*^{-/-} and *Nkx2.5*^{-/-} *Nkx2.6*^{-/-} embryos, expression of Shh in the pharynx was more restricted but was still detectable (Fig. 4I).

Phenotype of *Nkx2.5*^{-/-} *Nkx2.6*^{+/-} embryos. We crossed *Nkx2.5*^{+/-} *Nkx2.6*^{-/-} mice and *Nkx2.5*^{+/-} *Nkx2.6*^{+/-} mice to examine the phenotype of *Nkx2.5*^{-/-} *Nkx2.6*^{+/-} mice. Expectedly, the *Nkx2.5*^{-/-} *Nkx2.6*^{+/-} mutation was embryonically lethal. However, interestingly, *Nkx2.5*^{-/-} *Nkx2.6*^{+/-} embryos at E8.5 and E9.5 exhibited the same phenotype in the pharynx as *Nkx2.5*^{-/-} *Nkx2.6*^{-/-} embryos (Fig. 5A). Expression patterns of HNF3 β , Shh, and Pax9 were same as those in *Nkx2.5*^{-/-} *Nkx2.6*^{-/-} embryos (Fig. 5B to D). This result indicated that one copy of *Nkx2.6* in the absence of *Nkx2.5* was not sufficient for the proper formation of the pharynx.

Cardiac phenotype of *Nkx2.5*^{-/-} *Nkx2.6*^{-/-} embryos. In the course of analyzing mutant embryos, we noticed a difference in heart morphology in *Nkx2.5*^{-/-} *Nkx2.6*^{-/-} embryos from

Nkx2.5^{-/-} embryos. As described previously, the heart of *Nkx2.5*^{-/-} embryos lacked endocardial cushion formation but still had a cleft between the common atrium and ventricle (9, 20) (Fig. 1D). Notably, the distinction between the atrium and ventricle was less clear and the expansion of the common atrium was significantly less extensive in the heart of *Nkx2.5*^{-/-} *Nkx2.6*^{-/-} embryos (Fig. 1E). *Nkx2.5*^{-/-} *Nkx2.6*^{+/-} embryos showed the same cardiac phenotype (data not shown).

We next investigated alterations in cardiac gene expression in *Nkx2.5*^{-/-} *Nkx2.6*^{-/-} embryos. Expression of ANF and BNP was observed in both the atrium and ventricle of wild-type embryos (Fig. 6A and C). In *Nkx2.5*^{-/-} embryos, although expression of these genes in the ventricle was abolished, the atrial expression of ANF and BNP was still maintained (20). Interestingly, in *Nkx2.5*^{-/-} *Nkx2.6*^{-/-} embryos, expression of ANF and BNP in the atrium was also down-regulated. These genes were expressed only in a restricted region close to the atrioventricular canal (Fig. 6B and D). To assess the specification of the atrium and ventricle in *Nkx2.5*^{-/-} *Nkx2.6*^{-/-} embryos, we examined expression of MLC2A and MLC2V. In *Nkx2.5*^{-/-} *Nkx2.6*^{-/-} embryos, MLC2A was expressed in the atrium and ventricle and MLC2V was expressed only in the ventricle, albeit at a lower level, similar to results for *Nkx2.5*^{-/-} embryos (20) (Fig. 6E to H). This result indicated that the differentiation of atrial myocytes was less advanced but

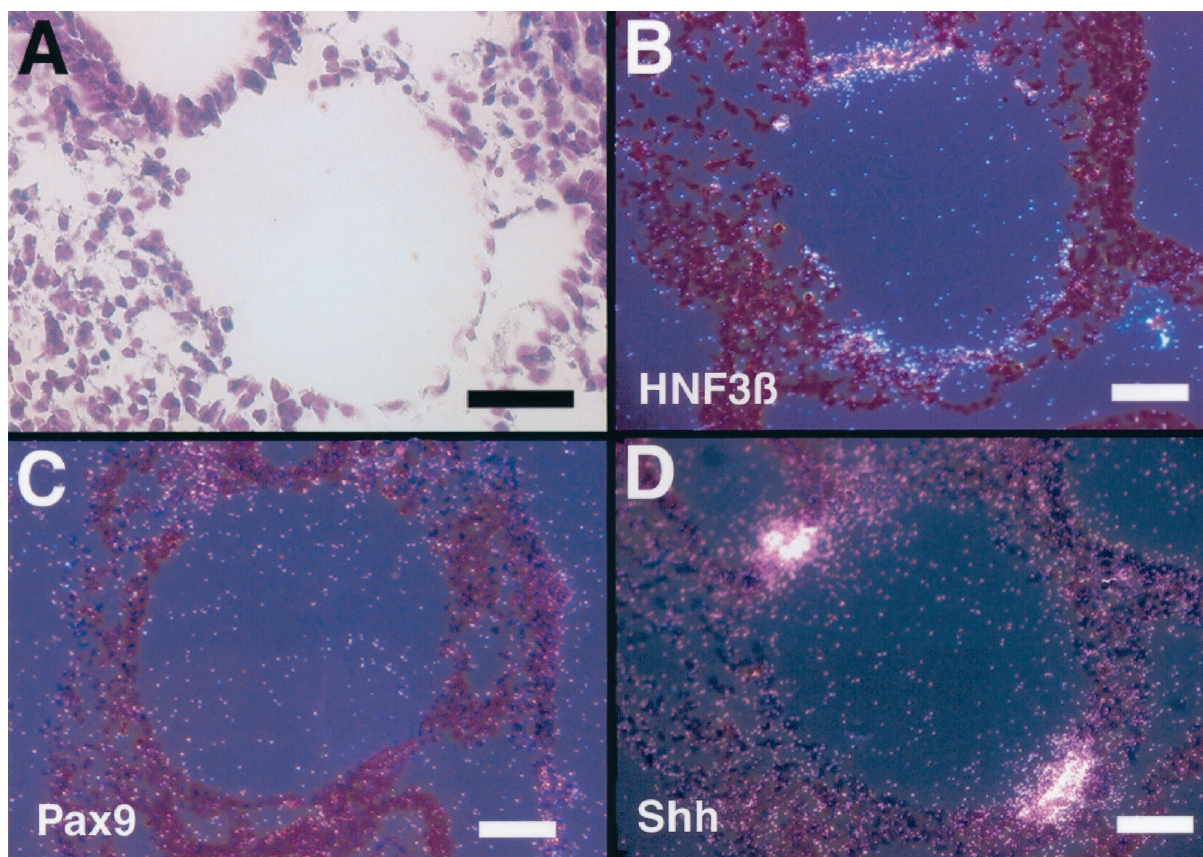


FIG. 5. Histological analysis of the pharynxes of $Nkx2.5^{-/-} Nkx2.6^{+/-}$ embryos. (A) Hematoxylin and eosin staining of a transverse section at E8.5. (B to D) In situ hybridization of transverse sections of $Nkx2.5^{-/-} Nkx2.6^{+/-}$ embryos at E8.75 using probes for the indicated proteins. Bars, 100 μm .

that the specification of the atrium and ventricle occurred normally in $Nkx2.5^{-/-} Nkx2.6^{-/-}$ embryos.

DISCUSSION

In this study, we demonstrated critical roles of the NK-2 homeobox genes $Nkx2.5$ and $Nkx2.6$ in pharyngeal development using a genetic approach. At the beginning of the embryonic period, cephalocaudal and lateral folding of the embryo incorporates the dorsal part of the endoderm-lined yolk sac into the embryo to form the primitive gut. The pharyngeal gut, or the pharynx, is the cephalic region of the primitive gut, extending from the buccopharyngeal membrane to the tracheobronchial diverticulum. The internal aspect of the pharynx is lined with epithelial endodermal cells, and a number of diverticula called pharyngeal pouches appear along the lateral wall of the pharynx (10). Although *hoxa3* and *Pax9* have been shown to be required for the normal development of pharyngeal pouch derivatives (4, 15), little is known about molecular mechanisms controlling pharyngeal development.

The ectopic expression of $Nkx2.5$ in pharyngeal pouches of $Nkx2.6^{-/-}$ embryos suggested a compensatory function of $Nkx2.5$ (22). Interestingly, in the double-mutant embryos, pharyngeal pouches did not form. Therefore, it is possible that inactivation of both $Nkx2.5$ and $Nkx2.6$ has eliminated overlapping functions of these genes in pharyngeal pouches. In the

pharyngeal floors of $Nkx2.5^{-/-}$ embryos, the numbers of endodermal cells were reduced and significantly fewer PCNA-positive cells were observed, indicating a critical role of $Nkx2.5$ in proliferation of pharyngeal endodermal cells. Moreover, there were only a few endodermal cells remaining in the pharynxes of $Nkx2.5^{-/-} Nkx2.6^{-/-}$ embryos, revealing overlapping functions of $Nkx2.5$ and $Nkx2.6$ in survival of pharyngeal endodermal cells. However, neither of these genes is expressed in the pharyngeal roof. Endodermal cells expressing $Nkx2.5$ and $Nkx2.6$ on the ventral side of the pharynx may secrete a signal molecule(s) essential for the maintenance of pharyngeal endodermal cells. Taken together, the data show that pharyngeal endodermal cells were induced and specified but could not proliferate and survive in the absence of $Nkx2.5$ and $Nkx2.6$. The abnormal dilatation of the pharynx could be due to the absence of the epithelial lining in the mutant pharynx. A small number of nonapoptotic cells expressing *Shh* in the ventral regions of $Nkx2.5^{-/-} Nkx2.6^{-/-}$ embryos remain to be characterized. These cells may derive from a different cell lineage from that of other pharyngeal epithelial cells.

The severe down-regulation of ANF and BNP expression in the atrium suggested that the cardiac program in the atrium is less advanced in $Nkx2.5^{-/-} Nkx2.6^{-/-}$ embryos, although the specification of the atrium and ventricle appeared to be nor-

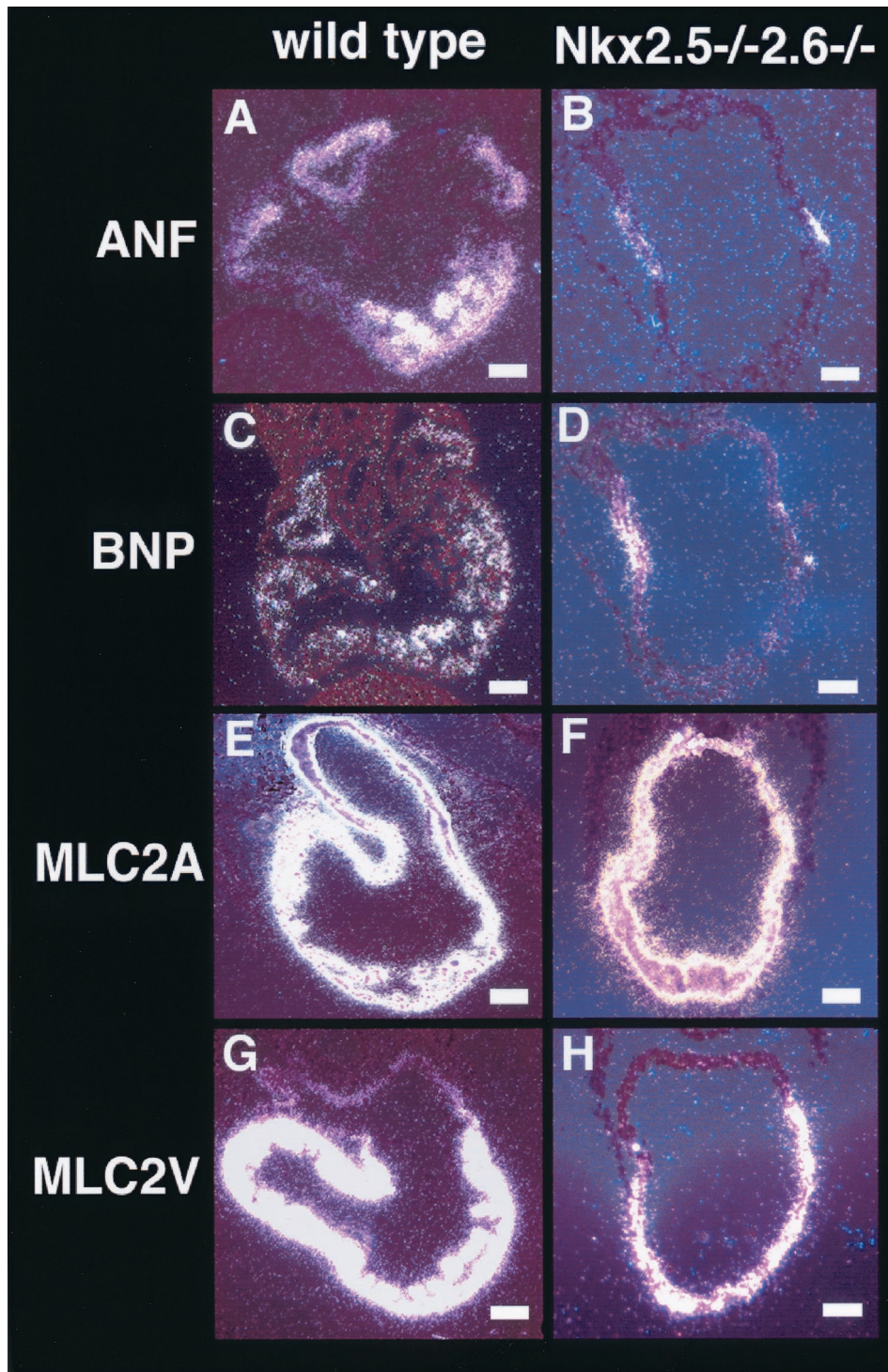


FIG. 6. Expression of cardiac tissue-specific genes. In situ hybridization of transverse sections of wild-type and $Nkx2.5^{-/-} Nkx2.6^{-/-}$ embryos at E8.75 using probes for the indicated proteins. Bars, 100 μ m.

mal. Since Nkx2.6 is not expressed in the atrium, it is possible that the absence or down-regulation of a secreted factor(s) from the pharyngeal endoderm might have caused this cardiac abnormality. This hypothesis is in line with previous studies showing that signals from the pharyngeal endoderm were re-

quired for differentiation of cardiac myocytes as well as specification of the cardiac cell lineage (11, 18, 19).

Interestingly, $Nkx2.5^{-/-} Nkx2.6^{+/-}$ embryos showed the same phenotype as $Nkx2.5^{-/-} Nkx2.6^{-/-}$ embryos. A similar dose-dependent requirement of transcription factors was

shown in the developmental program of the skeletal muscle. Gene targeting experiments showed that *Myf-5*^{+/-} *MyoD*^{-/-} as well as *Myf-5*^{-/-} *MyoD*^{-/-} mutations were lethal, implying that two functional copies of *Myf-5* were required to rescue *MyoD*^{-/-} mice (16).

In *Caenorhabditis elegans*, there is no heart, but pharyngeal muscle is of mesodermal origin and contracts rhythmically like vertebrate cardiac muscle. Moreover, *Ceh22*, a homolog of tinman in *C. elegans*, is expressed in the pharyngeal muscle and transactivates several pharynx specific genes (13, 14). Thus, it has been suggested that functions and development of pharyngeal muscle in *C. elegans* may be similar to those of cardiac muscle in higher organisms. The result that the murine homologs of tinman were critical for both pharyngeal and cardiac development support the hypothesis that the vertebrate heart and pharynx may share a common developmental program.

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