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SHORT COMMUNICATION

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NKX2 gene expression in neuroectoderm but not in mesendodermally derived structures depends on sonic hedgehog in mouse embryos

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Abstract NKX2 genes in vertebrates encode a subfamily of homeodomain-containing transcription factors which regulate morphogenetic events and cell differentiation during embryogenesis. In mouse embryos several NKX2 genes are expressed in the ventral midline domains of the neuroectoderm, while other NKX2 genes are primarily expressed in the mesendoderm and mesendodermally derived organs, such as heart and gut. Within several patterning centers for tissue organization sonic hedgehog (Shh) is an important signal in the formation of ventral midline structures in vertebrate embryos. Here, we investigated the role of Shh in the embryonic expression of six different but closely related NKX2 genes in Shh null mutant mice. We found that expression of NKX2.1, NKX2.2, and NKX2.9 in neural domains requires Shh signaling, whereas NKX2.3, NKX2.5 and NKX2.6 expression in endoderm and mesoderm is independent of Shh.

Key words NKX2 gene expression · Sonic hedgehog mutant · Mouse development

Introduction

NKX genes of vertebrates are divided into subgroups according to their structural homology to four Drosophila NK genes. Six NK2/3-related genes have been identified in mouse and shown to be active during embryonic development and in the adult (for review see Harvey 1996). Some of the murine NKX2 genes are expressed primarily in the developing brain and neural tube, while others are transcribed in branchial arches and in mesendoderm giving rise to heart and gut. As many of the NKX2 expression domains in mouse embryos appear to be confined to the ventral midline regions, we wondered whether the ventralizing signal Shh may be involved in activating these genes. To determine the possible role of Shh in NKX2 gene expression in mouse, we examined homozygous Shh null mouse embryos (Chiang et al. 1996) by whole mount in situ hybridization with various NKX2 probes and compared the patterns with wild-type embryos of corresponding developmental stages.

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Materials and methods

E9.5 wild-type and homozygous Shh mutant mouse embryos were fixed in 4% paraformaldehyde/phosphate-buffered saline at 4°C overnight and subjected to whole mount in situ hybridization as described previously (Bober et al. 1994). Riboprobes for Shh, NKX2.2, NKX2.9, and NKX2.3 were generated as described by Pabst et al. (1997, 1998). NKX2.5 probe was made from the entire cDNA coding region. The NKX2.6 template was produced from cDNA of branchial arches from an E9.5 mouse embryo by reverse transcription-polymerase chain reaction with the following primers: upstream primer, ATGCTGTCGAGTCCTGTGGC; downstream primer, GGCTCGCATAGCTAGCGTCG. The cDNA template for the NKX2.1 probe (provided by R. Di Lauro) encoded nucleotides 1763–2291 of the rat TTF-1 cDNA (Guazzi et al. 1990).

Results and discussion

We and others have previously shown that many of the NKX2 genes in vertebrate embryos are expressed in patterns that either overlap or abut domains of Shh expression. These observations raise the possibility that NKX2 genes are generally involved in cell-type specification in response to Shh signaling. To test this hypothesis we performed in situ hybridizations on homozygous Shh mouse mutants with various NKX2 probes. Representative re-

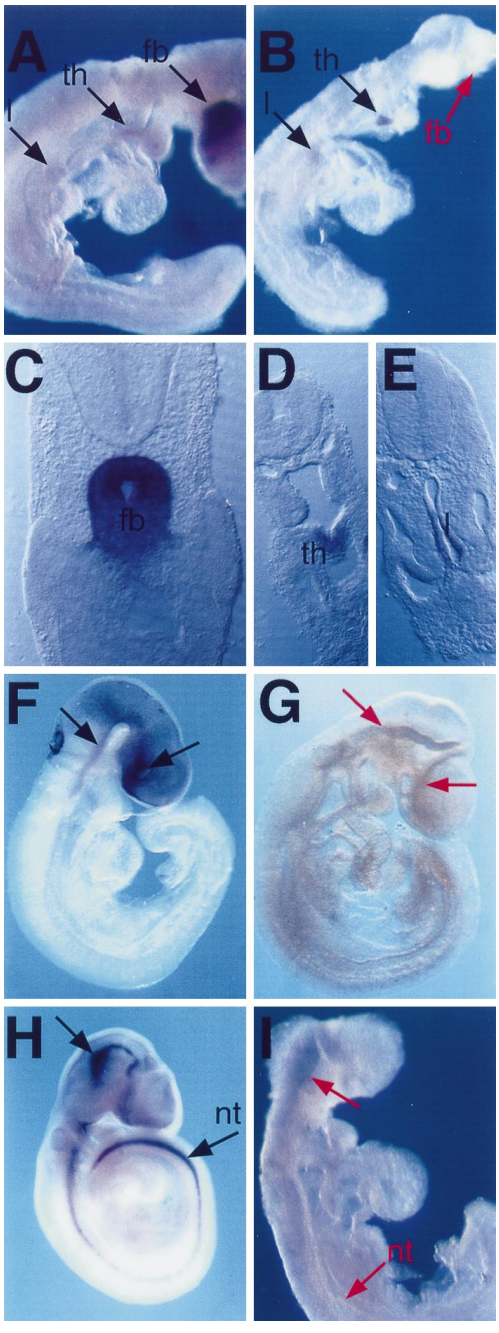

Fig. 1 Whole mount in situ hybridization and sections of E9.5 wild-type (**A**,**C**,**F**,**H**) and Shh mutant embryos (**B**,**D**, **E**, **G**, **I**) with NKX2.1 probe (**A**–**E**), NKX2.2 probe (**F**,**G**), and NKX2.9 probe (**H**,**I**). Expression domains in brain, thyroid and lung anlage, and neural tube are marked by *black arrows*. Expression missing in Shh mutant embryos is highlighted by *red arrows*. Transversal sections of the embryo shown in **A** and **B** are depicted in **C**, and **D** and **E**, respectively. NKX2.1 expression is shown in ventral forebrain of a wild-type mouse (**C**), and in the thyroid (**D**), and lung anlage (**E**) of mutant mice. The weak expression of NKX2.1 in lung and thyroid of the mutant mouse is unaltered compared to the wild type (data not shown) (*fb* forebrain, *th* thyroid gland, *l* lung, *nt* neural tube)

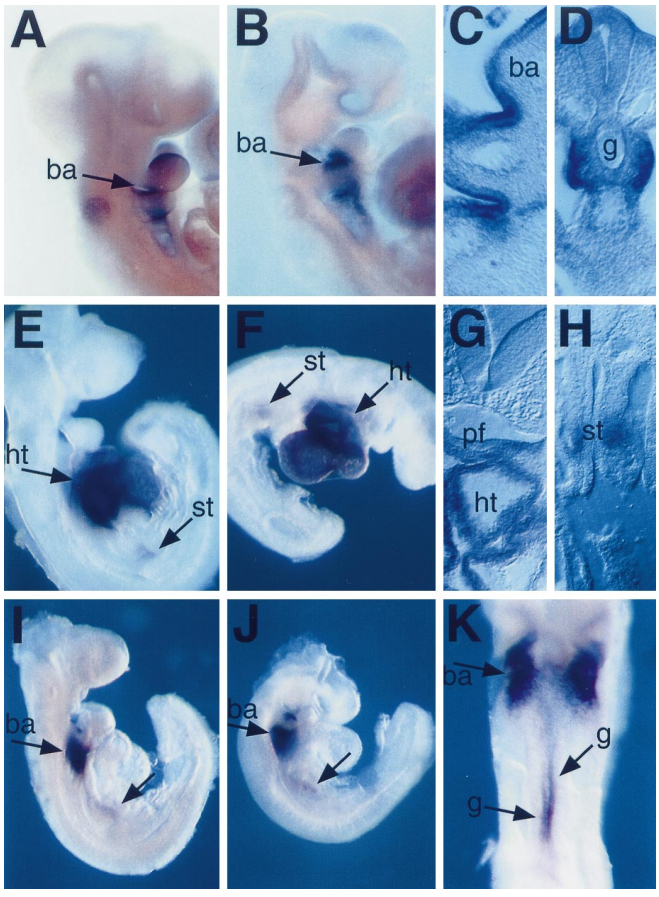

Fig. 2 Whole mount in situ hybridizations and sections of E9.5 wild-type (**A**,**E**,**I**) and Shh mutant embryos (**B**–**D**,**F**–**H**,**J**,**K**) with NKX2.3 (**A**–**D**), NKX2.5 (**E**–**H**) and NKX2.6 probes (**I**–**K**). Note that all expression domains (marked by *arrows*) in Shh mutants are unaltered as compared to wild type. Sections illustrate NKX2.3 expression in the branchial arch epithelium (**C**) and gut mesoderm (**D**), and NKX2.5 transcripts in myocardium, pharyngeal endoderm (**G**) and in the pylorus (**H**). NKX2.6 expression is detected in branchial arches and gut endoderm (**I**–**K**). The NKX2.6 transcript level appears to be slightly higher in a larger domain on the *left* side of the gut (**K**) (*ba* branchial arches, *g* gut, *ht* heart, *st* stomach, *pf* pharyngeal floor)

sults of embryonic NKX2 gene activities are illustrated for E9.5 mouse embryos (Figs. 1 and 2). In wild-type embryos transcripts for NKX2.1 accumulate in ventral forebrain (Fig. 1A, C), and in thyroid and lung anlagen (Fig. 1A). NKX2.2 mRNA is present also in forebrain and at low level in ventral hindbrain and neural tube (Fig. 1F). NKX2.9 is expressed in the brain and in ventral domains along the entire neural tube (Fig. 1H). These patterns appear in perfect agreement with previously published data (Guazzi et al. 1990; Price et al. 1992; Pabst et al. 1998). In contrast to wild-type embryos, E9.5 Shh null mutant embryos lack expression domains for all three genes in the neuroectoderm (red arrows in Fig. 1B, G, I), while NKX2.1 expression in the thyroid gland and lung is not affected by the Shh mutation (black arrows in Fig. 1B, D, E). The same results were obtained for other developmental stages (data not

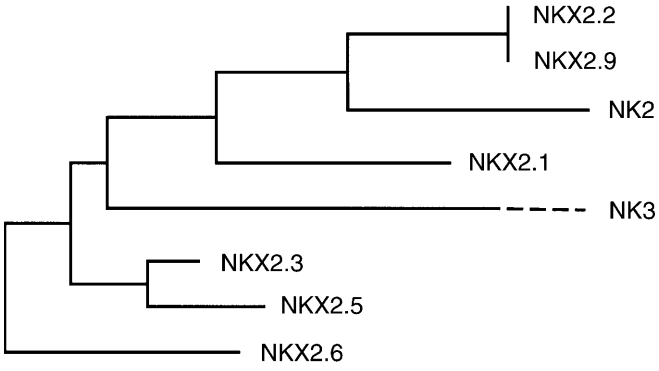

Fig. 3 Dendrogram of mouse NKX2 genes and their Drosophila homologs. The NKX genes, which are expressed in neuroectoderm and show dependence on Shh, form one subgroup (NKX2.1, NKX2.2, and NKX2.9), while the Shh-independent NKX genes form another subgroup (NKX2.3, NKX2.5 and NKX2.6). The relationship to Drosophila NK2 and NK3 genes is also shown. Sequences for this dendrogram were taken from Genbank database under the following accession numbers: NKX2.1, no. U19755; NKX2.2, no. U31566; NKX2.9, no. Y15741; NKX2.3, no. Y11117; NKX2.5, no. X75415; NKX 2.6, no. AF 045150; NK2, no. X87141; and NK3, no. L17133

shown), suggesting that Shh is absolutely required to activate these genes during embryonic development. Previous work has demonstrated that early telencephalic tissue responds to Shh signaling by expressing NKX2.1 (Ericson et al. 1995). Moreover, Pera and Kessel (1997) have recently shown that Shh expressed in prechordal plate of chick embryos is one of the signals involved in NKX2.1 gene activation in the ventral forebrain. NKX2.2 expression in embryonic forebrain of Zebrafish also appears responsive to Shh signals (Barth and Wilson 1995). A recent paper demonstrates that NKX2.2 has an essential role in interpreting graded Shh signals for the specification of neuronal identity in the ventral neural tube (Briscoe et al. 1999). Our results in the Shh mouse mutant confirm that all neuroectodermal domains of depend NKX2.1 and NKX2.2 gene expression on Shh and we extend these findings to the additional neural marker gene NKX2.9.

Unlike NKX2.1, NKX2.2, and NKX2.9, mouse NKX2.3, NKX2.5, and NKX2.6 are not expressed in neural structures, but are expressed in mesendodermal progenitor cells of heart and gut as well as in both mature organs throughout mouse development (Pabst et al. 1997; Lints et al. 1993; Biben et al. 1998). Regionalization of the gut involves epithelial-mesenchymal signaling, and Shh has been implicated as one of the signals (Roberts at al. 1998). Moreover, Shh mouse mutants display defects in foregut development (Litingtung et al. 1998), suggesting that Shh may affect at least some of the mesodermally expressed NKX2 genes. In E9.5 wild-type mouse embryos NKX2.3 is expressed in the epithelium of branchial arches and in mid- and hindgut mesoderm (Fig. 2A, and Pabst et al. 1997). The same spatial NKX2.3 expression is seen in $Shh^{-/-}$ mutant embryos of corresponding stages (Fig. 2B–D). NKX2.5 is highly transcribed in the pharyngeal floor, in the developing heart, and in the prospective pylorus (Fig. 2E). No alterations of NKX2.5 expression are observed in the Shh mutants (Fig. 2F–H). Likewise, NKX2.6 is expressed in branchial arch epithelium and gut endoderm at the level of the liver anlagen in both, wild-type and Shh mutant embryos (Fig. 2I–K). Significantly, NKX2.6 transcripts appear to accumulate asymmetrically in the pharyngeal endoderm with stronger expression on the left side, which is also maintained in the Shh mutant (Fig. 2K). Thus, no differences in the expression levels or regions of these NKX2 genes are seen between wild-type and Shh null mutant mice, indicating that Shh is not required for their activation. By inference, it can be concluded that these NKX genes, although expressed in domains with distinct boundaries along the antero-posterior gut axis, do not execute Shh-mediated patterning in the gut. They could, however, be part of a pre-patterning mechanism that leads to a regionalized response to the inductive influence of Shh in fore-, mid-, and hindgut (Roberts et al. 1998). In summary, one can distinguish two subgroups of NKX2 genes in mouse which are differentially dependent on Shh signaling. All expression domains of NKX2 genes in neuroectoderm require Shh, while all other sites of NKX2 gene activity are Shh independent.

Interestingly, the subdivision of NKX2 genes based on their expression is also reflected by their structural homology. Dendrograms compiled by sequence comparisons of the NKX2 homeodomains or the entire coding regions reveal that the Shh-dependent genes are more closely related to each other than to the members of the subgroup of Shh-independent NKX2 genes (Fig. 3). This observation may indicate that not only the structural properties of mouse NKX2 genes have been conserved after gene amplification but also at least some aspects of their expression control in the ventral domains of forebrain and neural tube.

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