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NK-2 class homeobox genes and pharyngeal/oral patterning: *Nkx2-3* is required for salivary gland and tooth morphogenesis

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ABSTRACT Head development in vertebrates requires reciprocal patterning interactions between cranial neural crest and the ectodermal, mesodermal and endodermal components of the branchial arches. Patterning elements within the pharyngeal endoderm and oral ectoderm appear to play defining roles in this process. Several homeobox genes of the *NK-2* class *(Nkx2-1, Nkx2-3, Nkx2-5* and *Nkx2-6)* are expressed regionally in the developing pharynx, and *Nkx2-1* mutants and *Nkx2-5/Nkx2-6* double mutants show loss of thyroid and distal lung progenitors, and pharyngeal cell viability, respectively. Here we examined the expression and genetic role of *Nkx2-3* in pharyngeal development. *Nkx2-3* was expressed in the pharyngeal floor and pouches, as well as in oral and branchial arch ectoderm. Expression persisted in the developing thyroid until birth, in mucous-forming cells of the lingual and sublingual salivary glands, and in odontogenic epithelium of the mandible. Examination of *Nkx2-3* null mice revealed defects in maturation and cellular organisation of the sublingual glands. Furthermore, cusps were absent from mandibular molars and the third molar was occasionally missing. These data suggest roles for *Nkx2-3* during pharyngeal organogenesis, although the considerable potential for genetic redundancy within and outside of this gene family may mask earlier functions in organ specification.

KEY WORDS: *Nkx2-3, homeobox, pharynx, salivary gland development, tooth development*

Introduction

It is a widely held view that the evolution of a mesenchymal neural crest facilitated formation of the complex vertebrate head (Shimeld and Holland, 2000). During head development, cranial neural crest cells migrate from the rhombomeric region of the hindbrain and populate the branchial arches, segmental structures that constitute both the physical and evolutionary scaffold for head formation. Historically, grafting experiments in chick embryos suggested that neural crest was the source of instructive patterning influences determining the unique developmental fate of branchial arches (Noden, 1983). More recent data, however, show that neural crest is considerably more plastic than once believed and that endoderm, ectoderm and cranial mesoderm are likely to provide principal patterning cues (Graham and Smith, 2001; Trainor and Krumlauf, 2001; Tucker *et al.,* 1998).

Anterior/posterior patterning in the pharyngeal endoderm may be controlled, at least in part, by retinoic acid (RA) signalling and *Hox* gene expression. A transgenic LacZ reporter gene controlled by a RA-responsive cis-element (RARE) is active in the caudal pharynx (Wendling *et al.,* 2000), and dietary, genetic or pharmacological inhibition of RA signalling leads to branchial arch agenesis and anterior homeotic-like shifts in caudal pharyngeal identity (Maden *et al.,* 1996; Niederreither *et al.,* 2001; Wendling *et al.,* 2000). Conversely, caudal homeotic-like shifts in the anterior pharynx have been observed after treatment of embryos with exogenous RA (Lee *et al.,* 1995; Marshall *et al.,* 1992). *Hoxa1* and *Hoxb1* are expressed in the caudal pharynx under RARE control and RA signalling blockade abolishes or reduces expression (Wendling *et al.,* 2000). *Hoxa3* is expressed in pharyngeal pouches 3 and 4 which contain precursors of the thymus, parathyroid and ultimobranchial body, and the gene is essential for specification and migration of these organs (Manley and Capecchi, 1998). In pharyngeal pouch development, *Hoxa3* interacts genetically with its paralogues *Hoxb3* and *Hoxd3*, as well as with *Pax1*, a paired homeodomain gene which, together with its relative *Pax9*, plays an essential role in pouch cell proliferation (Manley and Capecchi, 1998; Peters *et al.,* 1998; Su *et al.,* 2001).

Abbreviations used in this paper: BMP, bone morphogenetic protein; FGF, fibroblast growth factor; LacZ, β-galactosidase; PAS, periodic acid-Schiff; RA, retinoic acid; RARE, retinoic acid response element.

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Homeobox genes of the *NK-2* class (Harvey, 1996; McMahon, 2000) also play key roles in pharyngeal development. Within this class, two functionally distinct evolutionary clades are discernible (Harvey, 1996; Ranganayakulu *et al.,* 1998). A "neural" clade is expressed in the central nervous system and in mammals contributes to a transcription factor code specifying neuronal cell identity in the ventral spinal chord (McMahon, 2000). "Cardiac" genes are involved in patterning and differentiation of the heart and visceral mesoderm (Harvey, 1996; Lyons *et al.,* 1995; Wang *et al.,* 2000). Members of both clades are also expressed in restricted and overlapping domains in pharyngeal endoderm. The neural member *Nkx2-1* is essential for formation and differentiation of the thyroid anlagen and distal lung endoderm (Kimura *et al.,* 1996; Kimura *et al.,* 1999; Pasca di Magliano *et al.,* 2000; Yuan *et al.,* 2000), while cardiac genes *Nkx2-5* and *Nkx2-6* act inter-dependently and redundantly to maintain viability of pharyngeal cells (Tanaka *et al.,* 2001; Tanaka *et al.,* 2000).

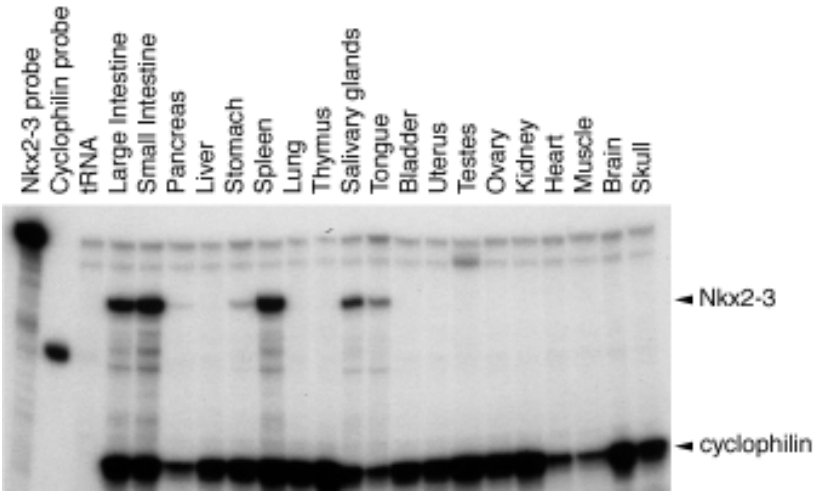

Fig. 1. RNase protection analysis of *Nkx2-3* expression. *The panel shows* Nkx2-3 *and control* cyclophilin *transcripts (arrowheads) detected by RNase protection in samples of total RNA extracted from adult tissues. Yeast tRNA was included as negative control.*

These mutant phenotypes and the restricted expression of *Nkx2* genes in the pharynx suggests that they play defining roles in pharyngeal regionalisation, and based on gene expression data an *Nkx2* homeobox gene code for determination of pharyngeal organs has been proposed (Reecy *et al.,* 1997). However, the mouse mutations have thus far provided no evidence for or against such a model. In this study, we examine the expression and genetic requirement for the cardiac clade member, *Nkx2-3,* in mouse pharyngeal development. In addition to its documented role in visceral mesoderm (Wang *et al.,* 2000), *Nkx2-3* was expressed broadly in embryonic pharyngeal floor endoderm extending laterally into the forming pharyngeal pouches and cranially into the oral cavity ectoderm. Expression largely overlapped that of its closest relatives *Nkx2-5* and *Nkx2-6* (Biben *et al.,* 1998; Lints *et al.,* 1993). Expression persisted in derivatives of the oral region, including tongue epithelium, salivary glands and odontogenic epithelium of the mandible. Examination of *Nkx2-3*$^{-/-}$ mutant mice showed normal specification but delayed differentiation and abnormal morphogenesis of the sublingual glands. Mandibular molars showed defects in cusp formation and the third molar was occasionally absent. The data principally suggest roles for *Nkx2-3* in differentiation of pharyngeal organ derivatives, although potentially redundant roles with other *Nkx2* genes in pharyngeal regionalisation and organ specification are discussed.

Results

Expression of Mouse Nkx2-3 *in Pharynx and Branchial Arches*

Expression of the *Nkx2-3* gene in pharyngeal endoderm and branchial arch ectoderm has been previously noted in studies on both mouse and chick embryos (Buchberger *et al.,* 1996; Pabst *et al.,* 1997). However, the onset and evolution of the expression patterns has not been described. To define the *Nkx2-3* transcript pattern in mouse in more detail, we first examined expression in adult tissues using RNase protection (Fig. 1). Expression was evident in stomach, spleen and intestine as previously documented (Wang *et al.,* 2000), and also in salivary glands and tongue, derivatives of the pharyngeal region. No expression, however, was seen in thymus or lung.

Expression during the early developmental period was assessed using *in situ* hybridisation of wholemount embryos (Fig. 2). Expression was first observed at early foregut pocket stages (~E7.75-E8.0) in ventral pharyngeal endoderm (Fig. 2 A,D). The pattern extended cranially to the buccopharyngeal membrane and caudally to a defined boundary inside the lip of the foregut pocket (Fig. 2D).

At E8.5, transverse sections of wholemount embryos at different cranial levels showed expression throughout the full medio-lateral extent of the ventral surface of the pharynx (Fig. 2 E-G). Anteriorly, transcripts were seen up to the level of the buccopharyngeal membrane continuing into the ectoderm of the oral cavity floor (Fig. 2H). Oral floor staining extended laterally into ectoderm associated with the cranial and dorsal part of the first pair of branchial arches (Fig. 2 G,H). Staining in ectoderm was also enhanced at a level just caudal to that of the first pharyngeal pouches, most likely defining the forming second arch (Fig. 2G). Additional weaker ectodermal staining was seen in regions lateral to the pharynx that will form more caudal arches (Fig. 2 E,F), although later, expression was only detected in first arch ectoderm.

At E9.5, *Nkx2-3* was expressed in the pharyngeal region in a pattern similar to that seen at E8.5 (Fig. 2 C, I-K). Expression in the foregut floor extended laterally into the pouches (Fig. 2I), cranially into the oral floor (Fig. 2K) and caudally to approximately the level of the future lung bud (Fig. 2 J,K).

Early Regionalisation of Nkx2 *Gene Expression in the Pharynx*

The role of RA in pharyngeal patterning in the mouse (see Introduction) is restricted to the 7-10 somite period (Wendling *et al.,* 2000), corresponding approximately to E8.5. The expression domains of some *Nkx2* genes became restricted in the anterior-posterior and medio-lateral axes of the pharynx at or even before this time. Three genes from of cardiac *Nkx2* clade (*Nkx2-3*, *Nkx2-5* and *Nkx2-6*) are expressed in the mouse pharynx (Biben *et al.,* 1998; Lints *et al.,* 1993; Pabst *et al.,* 1997). Initially, all are expressed in the full medio-lateral extent of the pharyngeal floor (Fig. 2 E-G, L; Biben *et al.,* 1998; Stanley *et al.,* 2002). However, *Nkx2-6* becomes restricted around E8.0 to the lateral regions encompassing the pharyngeal pouches (Fig. 2B), and thus becomes relatively downregulated in midline precursors of the lungs, thyroid, salivary glands and oral cavity (Biben *et al.,* 1998). Conversely, around the same time, *Nkx2-5* expression withdraws from

the pharyngeal pouches and becomes restricted to more midline regions (Fig. 2M; Lints *et al.,* 1993; Tanaka *et al.,* 2000). Notably, *Nkx2-5* expression is maintained in pharyngeal pouches of *Nkx2-6* mutant mice (Tanaka *et al.,* 2000), indicating a repressive interaction between the two genes. However, repression is not reciprocal, since the expression domain of *Nkx2-6* in the pharyngeal floor was not expanded in *Nkx2-5* mutant mice (not shown). The restriction of *Nkx2-6* expression to the pharyngeal pouches resembled that of *Pax9,* which is involved in pouch proliferation and survival (Peters *et al.,* 1998). However, a simple regulatory relationship between *Nkx2* and *Pax* genes seems precluded since *Pax9* was expressed more dorsally than any of the *Nkx2* genes (Fig. 2N).

Cranially, *Nkx2-3* and *Nkx2-5* are expressed up to the level of the buccopharyngeal membrane continuing into the oral floor ectoderm (Fig. 2 H,K,M; Stanley *et al.,* 2002). *Nkx2-6* is expressed in a similar fashion initially (Biben *et al.,* 1998; data not shown), but as noted above is rapidly down-regulated in midline regions of the pharyngeal floor, and thereafter the most cranial point of expression was the first pair of pharyngeal pouches (Fig. 2B; Biben *et al.,* 1998; Tanaka *et al.,* 2000). All three cardiac genes have a caudal boundary in the pharynx at a similar (although perhaps not identical) position, approximately the level of the future lung bud (Fig. 2 J,M; Biben *et al.,* 1998; Stanley *et al.,* 2002).

Only a single *Nkx2* gene of the neural clade, *Nkx2-1*, has thus far been reported to be expressed in the early pharynx (Lazzaro *et al.,* 1991). From the time of its activation around E8.0, expression of

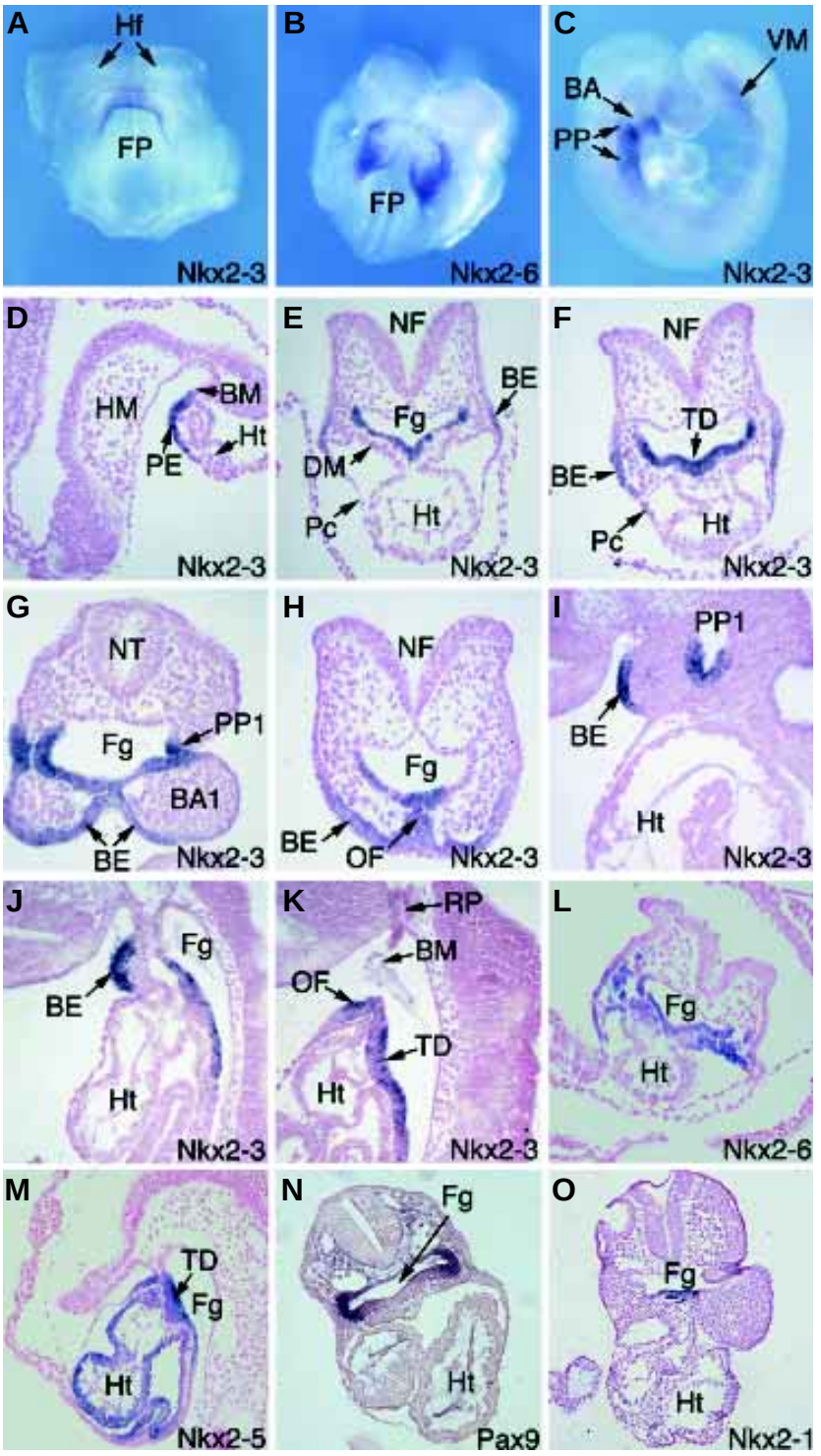

Fig. 2. Comparison of *Nkx2-3* expression with that of other regionally-expressed genes in the developing pharynx. *All panels show expression patterns detected by* in situ *hybridisation.* **(A)** *E8.0 wholemount embryo showing* Nkx2-3 *expression in ventral endoderm of the foregut pocket.* **(B)** *E8.25 embryo showing expression of* Nkx2-6 *restricted to lateral aspects of the foregut pocket endoderm. Note that slightly earlier,* Nkx2-6 *expression encompassed the entire pharyngeal floor (see panel N).* **(C)** *E9.0 embryo showing* Nkx2-3 *expression in the pharyngeal region and visceral mesoderm.* **(D)** *Section through an E8.25 embryo showing* Nkx2-3 *expression restricted to the endodermal layer.* **(E-H)** *Transverse sections of an E8.5 embryo at different cranial levels showing* Nkx2-3 *expression in pharyngeal floor endoderm and forming pharyngeal pouches. Expression in ectoderm of the oral floor and branchial arches is also highlighted. Panel E is the most caudal section and panel H is the most anterior section.* **(I-K)** *Sagittal (panel K) and parasagittal (panels I,J) sections through the pharyngeal region of an E9.0 embryo showing* Nkx2-3 *expression in pharyngeal floor endoderm up to the level of the buccopharyngeal membrane and oral floor ectoderm (panel K), as well as in first arch ectoderm (panels I,J). Note expression in a thickened region of the pharynx corresponding to the thyroid diverticulum (panel K).* **(L)** *Transverse section of an E8.0-8.25 embryo showing* Nkx2-6 *expression in pharyngeal floor endoderm and cranial mesoderm.* **(M)** *Parasagittal section of an E9.0 embryo showing* Nkx2-5 *expression in pharyngeal floor endoderm and heart mesoderm.* **(N)** *Transverse section of an E9.5 embryo showing* Pax9 *expression in dorsal and ventral aspects of the second pair of pharyngeal pouches.* **(O)** *Transverse section of an E9.0 embryo showing expression of* Nkx2-1 *restricted to the midline region of the pharynx. Abbreviations: BA, branchial arch; BE, branchial arch ectoderm; BM, buccopharyngeal membrane; DM, dorsal pericardial mesoderm; Fg, foregut; FP, foregut pocket; Hf, headfolds; HM, head mesoderm; Ht, heart; NF, neural folds; NT, neural tube; OF, oral cavity floor; Pc, pericardium; PE, pharyngeal endoderm; PP, pharyngeal pouch; RP, Rathke's pouch; TD, thyroid diverticulum; VM, visceral mesoderm.*

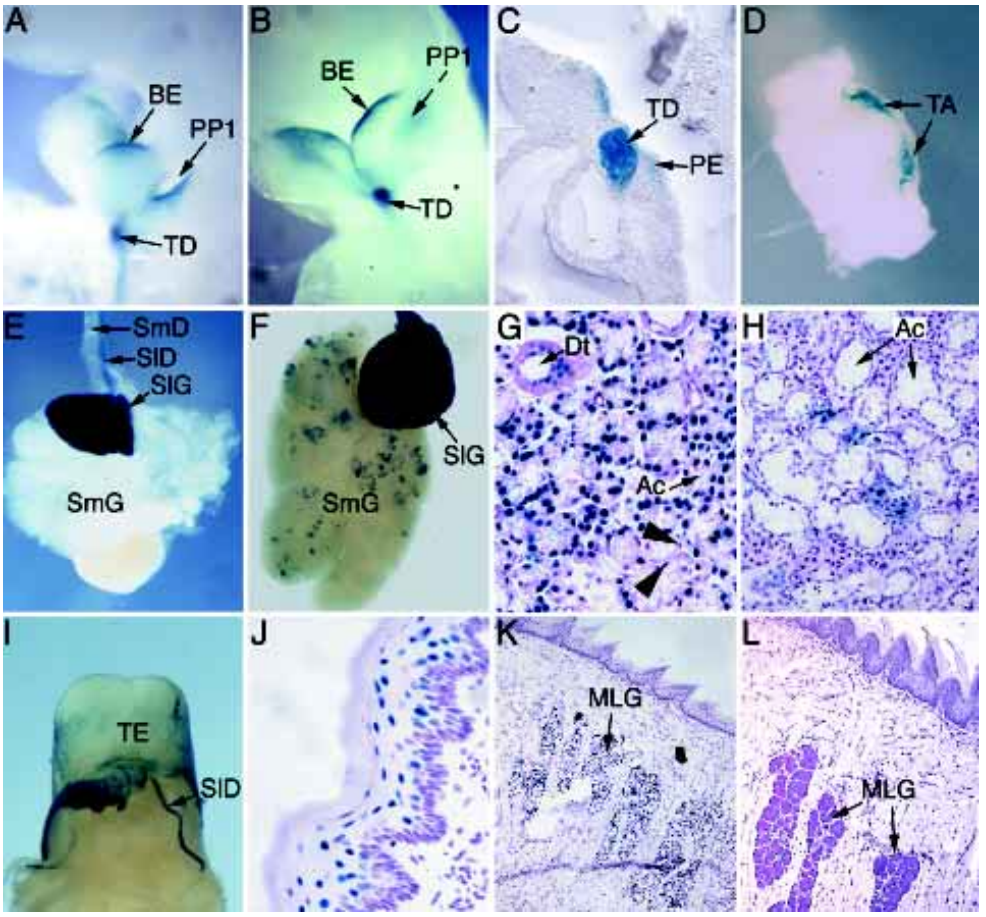

Fig. 3. Nkx2-3-LacZ expression in *Nkx2-3$^{lacZ\Delta HD/+}$* mice. *(A,B) Left-sided* **(A)** *and ventral* **(B)** *views of an E10.0 embryo showing LacZ expression in first branchial arch ectoderm, first pharyngeal pouch and thyroid diverticulum.* **(C)** *Sagittal section of an E10.0 embryo showing LacZ expression in pharyngeal floor endoderm with high levels in the thyroid diverticulum.* **(D)** *Dissected lower jaw (tongue removed) of an E13.5 embryo showing LacZ expression in tooth anlage.* **(E)** *Dissected adult submandibular and sublingual salivary glands with associated ducts from a subline of* Nkx2-3$^{lacZ\Delta HD}$ *mice in which the hygromycin resistance gene cassette has been retained (see text). LacZ expression is seen in the sublingual gland and its duct but not the submandibular gland.* **(F)** *Dissected adult submandibular and sublingual salivary glands from the* Nkx2-3$^{lacZ\Delta HD}$ *line in which the hygromycin resistance gene cassette has been removed (see text). Strong LacZ expression is seen in the sublingual gland and duct, with weak and sparse expression in the submandibular gland.* **(G)** *Histological section of an adult sublingual gland showing LacZ expression in nuclei of mucous acinar cell rosettes and ductal cells. Arrowheads indicate LacZ-negative cells within acini, most likely serous cells.* **(H)** *Section through submandibular gland shown in panel F highlighting LacZ staining in rare cells that appear to lie outside of the rosettes of mucous-secreting acini, which stain weakly with eosin.* **(I)** *Ventral view of a dissected adult tongue showing LacZ staining in tongue epithelium and major ducts of the sublingual gland.* **(J)** *Section through tongue epithelium showing LacZ staining only in nuclei of the post-mitotic differentiating epithelial layers.* **(K)** *Section through the dorsal part of the tongue showing LacZ-positive nuclei in structures that appear to be the minor lingual salivary glands. Tissue in this section was from a* Nkx2-3$^{lacZ\Delta HD/lacZ\Delta HD}$ *mouse.* **(L)** *Section through the dorsal part of a wildtype adult tongue stained with periodic acid Schiff reagent, which highlights mucous-secreting cells of the minor lingual salivary glands. Abbreviations: Ac, acinus; BE, branchial arch ectoderm; Dt, secretory duct; MLG, minor lingual salivary gland; PE, pharyngeal endoderm; PP, pharyngeal pouch; SID, sublingual duct; SIG, sublingual gland; SmD, submandibular duct; SmG, submandibular gland; TA, tooth anlage; TD, thyroid diverticulum; TE, tongue epithelium.*

Nkx2-1 occurred only in the midline of the pharynx, covering the thyroid and lung precursors (Fig. 2, image O).

Evolution of the Nkx2-3 *Expression Pattern in Pharyngeal and Oral Derivatives*

To examine the further evolution of *Nkx2-3* expression, we assayed β-galactosidase (LacZ) activity in embryos and adults of the *Nkx2-3$^{lacZ\Delta HD}$* strain of mice. In this strain, a nuclear LacZ expression cassette linked to an internal ribosome entry site was inserted by gene targeting into the second coding exon of the *Nkx2-3* locus (Wang *et al.,* 2000). LacZ expression in the pharyngeal region of *Nkx2-3$^{lacZ\Delta HD/+}$* mice at E10.0 was essentially identical to that detected earlier by *in situ* hybridization, with staining evident in the pharyngeal floor, first pharyngeal pouch and branchial arch ectoderm (Fig. 3A). This and previously published expression data (Pabst *et al.,* 1997; Wang *et al.,* 2000) strongly suggests that LacZ staining in the *Nkx2-3$^{lacZ\Delta HD}$* strain accurately reflects the endogenous *Nkx2-3* expression pattern (although see below).

At E10.0, LacZ expression in *Nkx2-3$^{lacZ\Delta HD/+}$*embryos was observed throughout the floor of the pharynx, with highest expression in the descending thyroid diverticulum (Fig. 3 A,B). Thyroid expression persisted throughout foetal life but waned towards birth and was undetectable in neonates. At E13.5, high LacZ expression was seen in mandibular odontogenic epithelium (Fig. 3C), but not in its maxillary counterpart. This pattern is consistent with the strong LacZ expression seen in the region of the odontogenic ectoderm in the first pair of branchial arches at E10.0 (Fig. 3A), but not in their maxillary processes at later stages (not shown).

In foetal and adult *Nkx2-3$^{lacZ\Delta HD/+}$* mice, strong LacZ expression was seen in the bilaterally-arranged sublingual salivary glands (Fig. 3 E,F). Weak and sparse LacZ expression was also evident in the associated submandibular glands (Fig. 3F), but the parotid glands were negative (not shown). Interestingly, the sparse submandibular gland expression was seen only in an *Nkx2-3$^{lacZ\Delta HD}$* line in which the LoxP-flanked hygromycin resistance cassette (a remnant of gene targeting procedures) had been removed using Cre recombinase (see (Wang *et al.,* 2000)), and not in a sister line in which it was retained (Fig. 3 E,F). This finding indicates some regulatory interference from cis-elements

within the hygromycin resistance cassette, although no other LacZ expression differences have been seen so far between the two lines.

The sublingual and submandibular glands develop within paired gutter-like grooves (the alveolo-lingual sulci) in the anterior pharyngeal floor endoderm formed as a result of upward growth of the tongue rudiment. Closure of the grooves also creates the major secretory ducts for these organs that open into the oral cavity more anteriorly. The role of the sublingual gland is predominantly to secrete mucous under parasympathetic control. Sectioning of glands after wholemount staining showed nuclear LacZ expression largely restricted to mucous-secreting acinar cells (Fig. 3G). Rarer LacZ-negative acinar cells were usually found in clusters (Fig. 3G) and may correspond to the serous cells also found in this gland (Young and van Lennep, 1978). These cells, generally arranged in terminal acinar structures called "demilunes", secrete a mixture of enzymes, antibodies and some mucous (Young and van Lennep, 1978). The major secretory ducts were LacZ-positive along their entire length (Fig. 3 E,I) and within the glands, ducts were composed of LacZ-positive and negative cells in an approximately 1:1 ratio (Fig. 3G). Neuronal cells, connective tissue fibroblasts, and vascular smooth muscle and endothelial cells within the glands did not express LacZ. In submandibular glands, the rare LacZ-positive cells have not been specifically identified, but do not appear to correspond to mucous-secreting cells, which stain poorly with eosin (Fig. 3H).

LacZ expression in adults was also seen in epithelium of the tongue (Fig. 3I), consistent with previous detection of *Nkx2-3* transcripts in tongue epithelium of E14.5 embryos (Pabst *et al.,* 1997). Expression was evident only in post-mitotic layers at the outer lingual surface (Fig. 3J). Within the body of the tongue, several clusters of LacZ-positive cells were also detected (Fig. 3K). The similarity in shape and position of these clusters with those staining positive for the periodic acid-Schiff (PAS) reaction, which highlights glycoproteins concentrated in mucous (Fig. 3L), suggests that they correspond to the minor mucous-secreting lingual salivary glands (Schneider *et al.,* 2000; Young and van Lennep, 1978).

Phenotypic Analysis of Nkx2-3$^{-/-}$ *Mice*

Nkx2-3$^{lacZ\Delta HD/lacZ\Delta HD}$ mice show ~30% lethality due to a complex intestinal malabsorption phenotype (Wang *et al.,* 2000). Survivors recover rapidly although show splenic and Peyer's patch hypoplasia, intestinal villus dysmorphogenesis, gut truncation and distension, and defects in lymphocyte homing. These phenotypes relate to the loss of *Nkx2-3* expression and function in the visceral mesoderm (Fig. 2C; Wang *et al.,* 2000).

We examined teeth and salivary glands in *Nkx2-3*$^{lacZ\Delta HD/lacZ\Delta HD}$ mutant mice, structures that showed persistant LacZ staining into adulthood (see above). While incisors appeared normal, the mandibular molars of mutants were clearly abnormal. They often failed to erupt completely from the gums, and their cusps were largely absent (Fig. 4 A,B). In most cases the structure of the tooth body and root system was normal (Fig. 4C), although the third molar was missing on one side of the jaw in 2 of 15 mice examined. All teeth of the upper jaw appeared normal, consistent with the lack of *Nkx2-3* expression in their precursors (see above).

The sublingual glands and ducts in *Nkx2-3*$^{lacZ\Delta HD/lacZ\Delta HD}$ mutant mice were of normal size. However, differentiation of mucous-secreting acinar cells was retarded and cellular architecture was disrupted. In adult glands, histological staining showed a lack of compression of acinar cell nuclei against the membrane of their outer basal surface, a feature that develops in mature glands due to the large volume occupied by secretory granules (Fig. 4 D,E) (Young and van Lennep, 1978). Furthermore, delayed maturation of mucous acinar cells could be detected in neonatal organs by

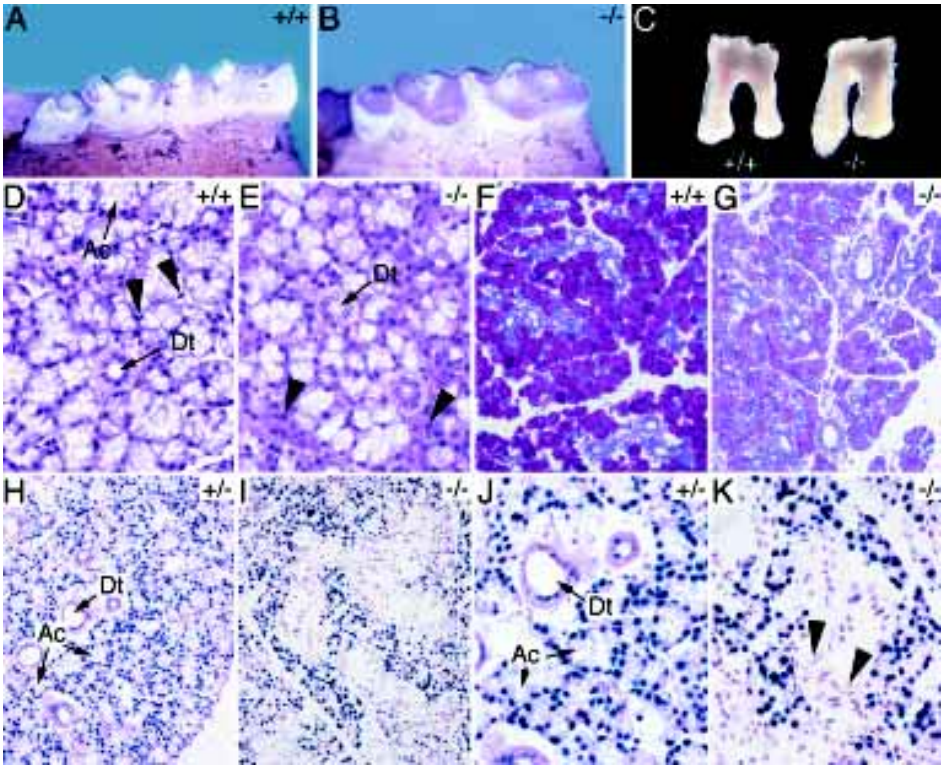

Fig. 4. Phenotypic analysis of *Nkx2-3*$^{lacZ\Delta HD/lacZ\Delta HD}$ mice. **(A,B)** *Mandibular molars from wildtype (A; +/+) and* Nkx2-3$^{lacZ\Delta HD/lacZ\Delta HD}$ *mutant (B; -/-) adult mice. In these samples, gums have been resected and teeth have been briefly stained with haematoxylin to highlight cusp profiles.* **(C)** *Dissected mandibular molars from wildtype and* Nkx2-3$^{lacZ\Delta HD/lacZ\Delta HD}$ *mutant mice showing normal tooth body and root formation in mutants.* **(D,E)** *Histological sections of adult wildtype and* Nkx2-3$^{lacZ\Delta HD/lacZ\Delta HD}$ *mutant sublingual glands stained with haematoxylin and eosin. Arrowheads in panel D indicate compressed nuclei within mucous acinar cells. Arrowheads in panel E show the excess of eosinophilic cells in the mutant gland.* **(F,G)** *Histological sections of neonatal wildtype and* Nkx2-3$^{lacZ\Delta HD/lacZ\Delta HD}$ *mutant sublingual glands stained with PAS reagents, showing retarded accumulation of mucous glycoproteins in the mutant gland. (H-K) Histological sections of young adult* Nkx2-3$^{lacZ\Delta HD/+}$ *(+/-) and* Nkx2-3$^{lacZ\Delta HD/lacZ\Delta HD}$ *(-/-) mutant sublingual glands at low* **(H,J)** *and high* **(I,K)** *magnification. Sections are stained for LacZ activity and counterstained with haematoxylin and eosin. Arrowheads in panel K indicate LacZ-negative cells staining poorly with haematoxylin, that appear to represent an expanded minor non-acinar cell population. Abbreviations: Ac, acinus; Dt, salivary gland duct.*

PAS staining (Fig. 4 F,G), which revealed lower levels of mucous glycoproteins in mutant glands. There was also a relative excess in mutant glands of eosinophilic cells surrounding the mucous-secreting acinar rosettes, which in contrast stain poorly with eosin (Fig. 4 D,E). In some mutant organs, there was a high degree of cellular disarray (Fig. 4 H-K). LacZ staining with histological counterstaining highlighted large areas of LacZ-negative cells, with relatively fewer LacZ-positive cells poorly organised into acinar structures. About one half of the LacZ-negative cells showed nuclei that stained very weakly with haematoxylin suggesting that they represent a minor non-acinar, precursor or mis-specified cell type.

Discussion

In this study we have examined the genetic requirement for the *NK-2* class homeobox gene, *Nkx2-3,* in pharyngeal development. Expression of *Nkx2-3* was initiated in pharyngeal floor endoderm at early foregut pocket stages, overlapping the window of sensitivity in the pharynx for disturbances to RA signalling, potentially mediated by modifications to *Hox* gene expression (Wendling *et al.,* 2000). The early pattern of *Nkx2-3* expression in the pharynx and oral ectoderm very closely matched that of its nearest relatives, *Nkx2-5* and *Nkx2-6.* However, these genes rapidly adopted reciprocal expression patterns, with *Nkx2-5* transcripts becoming restricted to the pharyngeal and oral midline and those of *Nkx2-6* to the pharyngeal pouches (Tanaka *et al.,* 2001; Tanaka *et al.,* 2000). Thus, at early stages, *Nkx2-3* is expressed in a pattern that very neatly matches the sum of the *Nkx2-5* and *Nkx2-6* patterns. These findings suggest complex regulatory interactions between members of this homeobox gene sub-family as well as considerable potential for redundant function. Indeed, *Nkx2-5* and *Nkx2-6* appear to act redundantly in maintaining viability of early pharyngeal endodermal cells (Tanaka *et al.,* 2001; Tanaka *et al.,* 2000).

Beyond the early stages of pharyngeal patterning, *Nkx2-3* expression was maintained in the developing thyroid (until birth), minor lingual and major sublingual salivary glands, tongue epithelium and mandibular odontogenic epithelium. In *Nkx2-3*$^{lacZ\Delta HD/lacZ\Delta HD}$ mice the thyroid appeared histologically normal. However, any function for *Nkx2-3* in the specification and/or ontogeny of the thyroid may be compensated for in mutants by *Nkx2-5* expression (Lints *et al.,* 1993). *Nkx2-3* and *Nkx2-5* are close evolutionary relatives (Harvey, 1996) and in frogs, dominant-negative inhibition experiments suggest that they play redundant roles in cardiogenesis (Grow and Krieg, 1998). The early survival of the thyroid anlagen and control of thyroid gene expression critically requires Nkx2-1 (Kimura *et al.,* 1996; Kimura *et al.,* 1999), in collaboration with Pax8, TTF-2 and CTF/NF-1 (Mansouri *et al.,* 1998; Ortiz *et al.,* 1999). The specific roles of *Nkx2-3* and *Nkx2-5* in thyroid development, in relation to those of *Nkx2-1*, remain to be determined.

We detected maturational defects in both tooth and salivary gland development in *Nkx2-3*$^{lacZ\Delta HD/lacZ\Delta HD}$ mice. The lower jaw molars of mutants were specified normally, although the third molar was occasionally missing. Furthermore, the cusps of formed molars were essentially absent. Tooth development occurs in response to complex and changing interactions between epithelial and mesenchymal populations in the first pair of branchial arches, involving signalling molecules of the FGF, BMP, sonic hedgehog and Wnt families and numerous transcription factors (Peters and Balling, 1999). A homeodomain transcription factor code for individual tooth identity has been proposed (Peters and Balling, 1999) and transformation of incisors into molars has been achieved *in vitro* by modulation of BMP signalling and thereby the relative expression domains of homeobox genes *Barx-1* and *Msx-1* (Tucker *et al.,* 1998). Antagonistic signalling by FGF and BMP ligands in oral ectoderm appears to determine the initial patterns of gene expression in mesenchyme that specify odontogenic potential and tooth identity (Peters and Balling, 1999; Tucker *et al.,* 1998). *Nkx2-3* was initially expressed broadly in ectoderm of the first pair of branchial arches, although by E10.0 had become restricted to paired narrow ectodermal stripes, presumably odontogenic epithelium. By E13.5, expression was seen in tooth anlage of the lower jaw, with highest levels in the molar regions. The mechanism of this restriction has not been defined, although we can reasonably expect, drawing on parallels from heart and visceral mesoderm (Harvey, 1996; Smith *et al.,* 2000; Sparrow *et al.,* 2000), a role for BMP signalling in induction or maintenance of the *Nkx2-3* pattern.

The individuality in the pattern of tooth cusps defines the identity of molars. Cusp formation is controlled by enamel knots, transient ectodermal signalling centres that regulate the balance between cell proliferation and cell death in the ectoderm, and subtle differences in these responses determines the final profile of each tooth (Peters and Balling, 1999). The loss of cusps in the lower molars of *Nkx2-3*$^{lacZ\Delta HD/lacZ\Delta HD}$ mice suggests a role for *Nkx2-3* in the latter stages of tooth morphogenesis, perhaps in regulating the proliferative response of ectoderm to FGFs expressed by mesenchyme (Peters and Balling, 1999). *Nkx2-3* could also be necessary for formation of enamel knots that orchestrate cusp morphogenesis. An alternative hypothesis is that terminal differentiation of ameloblasts is affected, leading to weak enamel which becomes eroded by mastication. The restriction in *Nkx2-3* expression and function to the lower jaw highlights the fact that different patterning mechanisms act in morphogenesis of mandibular and maxillary molars. This is also evident in mice lacking the homeogenes *Dlx1* and *Dlx2*, where only maxillary teeth are affected (Qiu *et al.,* 1997). The availability of a host of markers for the multiple stages of tooth development (Peters and Balling, 1999) will facilitate further analysis of this dichotomy.

The sublingual salivary glands in *Nkx2-3*$^{lacZ\Delta HD/lacZ\Delta HD}$ mice were specified normally, but showed retarded maturation. Furthermore, cellular architecture within the gland was variably disrupted and in some cases there was expansion of minor cell populations at the apparent expense of mucous acinar cells, in which *Nkx2-3* was expressed. This finding suggests that inductive interactions between *Nkx2-3*-positive and negative cells determine the cellular architecture of this gland. The minor lingual salivary glands appeared grossly normal in *Nkx2-3*$^{lacZ\Delta HD/lacZ\Delta HD}$ mutants.

The pharyngeal phenotypes expressed in *Nkx2-3*$^{lacZ\Delta HD/lacZ\Delta HD}$ mice suggest roles for *Nkx2-3* in differentiation of cell types within its organ derivatives. However, as for other genes expressed in regional patterns in the branchial region (Qiu *et al.,* 1997), potential redundancy between *Nkx2* members may mask earlier roles in regionalisation and thus specification of organ precursors within the pharyngeal floor. Indeed, the loss of the thyroid and distal lung in *Nkx2-1* mutants (Kimura *et al.,* 1996; Kimura *et al.,* 1999; Yuan *et al.,* 2000), and occasional loss of the third molar in *Nkx2-3* mutants supports this notion. Furthermore, an early role in viability of endodermal cells of the pharynx was revealed in mice doubly mutant for *Nkx2-5* and *Nkx2-6* (Tanaka *et al.,* 2001). It has been suggested that the collective stoichiometry of different *Hox* gene transcripts acting on common targets, rather than a *Hox* gene

code, is an essential feature of *Hox*-mediated patterning in the pharynx and other organs (Manley and Capecchi, 1998), and this may also be true of the early and late functions of *Nkx2* genes. In this light it is interesting that *Nkx3.1*, a more distant relative of *Nkx2-3*, is required for determining the size and architecture of the minor salivary glands (Schneider *et al.,* 2000). Even though they are not members of the same homeobox gene class, *Nkx3.1* and *Nkx2-3* may have similar as well as unique functions in salivary gland development. Sorting out early and later functions, as well as redundant and unique functions of *Nkx2* genes in the pharynx in relation to other patterning mechanisms, such as the RA/*Hox* system, is a challenging problem in craniofacial development. These goals will be greatly assisted by the respective targeted mutations and the capacity for culture and ex-vivo manipulation of pharyngeal arch explants.

Materials and Methods

RNase Protection

RNase protection analysis was performed as described (Ambion Instruction Manual RPA II; catalogue #1410) using RNase A (Sigma, catalogue #R5250) and RNase T1 (Sigma, catalogue #R1003). RNA probes were synthesised using T7 RNA polymerase (Promega). The *Nkx2-3* probe was synthesised from a template carrying a 237bp *Nar*I fragment corresponding to nucleotides 2620-2856 of the *Nkx2-3* genomic sequence (GenBank AF155583). The cyclophilin probe was synthesised from a template purchased from Ambion.

Wholemount In Situ *Hybridisation*

Wholemount *in situ* hybridisation was performed as described (Biben *et al.,* 1998; Lints *et al.,* 1993). Noon on the day of plugging was taken as embryonic stage E0.5. The *Nkx2-3* probe was synthesised from a template carrying a 643 bp *Hin*fI/*Nco*I fragment corresponding to nucleotides 2688-3330 of the *Nkx2-3* genomic sequence (GenBank: AF155583). *Nkx2-1*, *Nkx2-5*, *Nkx2-6* and *Pax9* probes have been previously described (Biben *et al.,* 1998; Lazzaro *et al.,* 1991; Lints *et al.,* 1993; Peters *et al.,* 1998).

LacZ Staining and Histology

LacZ staining of wholemount embryos and tissues, and tissue sections was performed as described (Wang *et al.,* 2000). Sections were post-fixed with 4% paraformaldehyde for over 2 hours and were generally counterstained with haematoxylin and eosin (H and E). PAS staining was performed on paraffin-embedded tissue after sectioning using standard protocols.

Nkx2-3$^{lacZ\Delta HD}$ Mice

Nkx2-3$^{lacZ\Delta HD}$ mice were maintained as both heterozygous and homozygous strains and were on a mixed 129SvJ x C57BL/6 background.

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References

BIBEN, C., HATZISTAVROU, T. and HARVEY, R.P. (1998). Expression of *NK-2* class homeobox gene *Nkx2-6* in foregut endoderm and heart. *Mech. Dev.* 73:125-127.

BUCHBERGER, A., PABST, O., BRAND, T., SEIDL, K. and ARNOLD, H.-H. (1996). Chick NKx-2.3 represents a novel family member of vertebrate homologues to Drosophila homeobox gene tinman: Differential expression of cNKx-2.3 and cNKx-2.5 during heart and gut development. *Mech. Dev.* 56:151-163.

GRAHAM, A. and SMITH, A. (2001). Patterning the pharyngeal arches. *Bioessays.* 23:54-61.

GROW, M.W. and KRIEG, P.A. (1998). Tinman Function Is Essential for Vertebrate Heart Development: Elimination of Cardiac Differentiation by Dominant Inhibitory Mutants of the *tinman*-Related Genes, *XNkx2-3* and *XNkx2-5*. *Dev. Biol.* 204:187-196.

HARVEY, R.P. (1996). *NK-2* Homeobox Genes and Heart Development. *Dev. Biol.* 178:203-216.

KIMURA, S., HARA, Y., PINEAU, T., FERNANDEZ-SALGUERO, P., FOX, C.H., WARD, J.M. and GONZALEZ, F.J. (1996). The T/ebp null mouse: thyroid-specific enhancer-binding protein is essential for organogenesis of the thyroid, lung, ventral forebrain, and pituitary. *Genes Dev.* 10:60-69.

KIMURA, S., WARD, J.M. and MINOO, P. (1999). Thyroid-specific enhancer-binding protein/thyroid transcription factor 1 is not required for the initial specification of the thyroid and lung primordia. *Biochimie.* 81:321-327.

LAZZARO, D., PRICE, M., DE FELICE, M. and DI LAURO, R. (1991). The transcription factor TTF-1 is expressed at the onset of thyroid and lung morphogenesis and in restricted regions of the foetal brain. *Development.* 113:1093-1104.

LEE, Y.M., OSUMI-YAMASHITA, N., NINOMIYA, Y., MOON, C.K., ERIKSSON, U. and ETO, K. (1995). Retinoic acid stage-dependently alters the migration pattern and identity of hindbrain neural crest cells. *Development.* 121:825-837.

LINTS, T.J., PARSONS, L.M., HARTLEY, L., LYONS, I. and HARVEY, R.P. (1993). *Nkx-2.5*: a novel murine homeobox gene expressed in early heart progenitor cells and their myogenic descendants. *Development.* 119:419-431.

LYONS, I., PARSONS, L.M., HARTLEY, L., LI, R., ANDREWS, J.E., ROBB, L. and HARVEY, R.P. (1995). Myogenic and morphogenetic defects in the heart tubes of murine embryos lacking the homeobox gene *Nkx2-5*. *Genes Dev.* 9:1654-1666.

MADEN, M., GALE, E., KOSTETSKII, I. and ZILE, M. (1996). Vitamin A-deficient quail embryos have half a hindbrain and other neural defects. *Current Biol.* 6:417-426.

MANLEY, N.R. and CAPECCHI, M.R. (1998). Hox Group 3 Paralogs Regulate the Development and Migration of the Thymus, Thyroid, and Parathyroid Glands. *Dev. Biol.* 195:1-15.

MANSOURI, A., CHOWDHURY, K. and GRUSS, P. (1998). Follicular cells of the thyroid gland require Pax8 gene function. *Nat. Genet.* 19:87-90.

MARSHALL, H., NONCHENV, S., SHAM, M.H., MUCHAMORE, I., LUMSDEN, A. and KRUMLAUF, R. (1992). Retinoic acid alters hindbrain Hox code and induces transformation of rhombomeres 2/3 into a 4/5 identity. *Nature.* 360:737-741.

MCMAHON, A. (2000). Neural patterning: The role of Nkx genes in the ventral spinal cord. *Genes Dev.* 14:2261-2264.

NIEDERREITHER, K., VERMOT, J., MESSADDEQ, N., SCHUHBAUR, B., CHAMBON, P. and DOLLE, P. (2001). Embryonic retinoic acid synthesis is essential for heart morphogenesis in the mouse. *Development.* 128:1019-1031.

NODEN, D. (1983). The role of the neural crest in patterning of avian cranial skeleton, connective, and muscle tissues. *Dev. Biol.* 96:144-165.

ORTIZ, L., AZA-BLANC, P., ZANNINI, M., CATO, A.C.B. and SANTISTEBAN, P. (1999). The Interaction between the Forkhead Thyroid Transcription Factor TTF-2 and the Constitutive Factor CTF/NF-1 Is Required for Efficient Hormonal Regulation of the Thyroperoxidase Gene Transcription. *J. Biol. Chem.* 274:15213-15221.

PABST, O., SCHNEIDER, A., BRAND, T. and ARNOLD, H.-H. (1997). The Mouse Nkx2-3 Homeodomain Gene is Expressed in Gut Mesenchyme During Pre- and Postnatal Mouse Development. *Dev. Dynamics.* 209:29-35.

PASCA DI MAGLIANO, M., DI LAURO, R. and ZANNINI, M. (2000). Pax8 has a key role in thyroid cell differentiation. *Proc. Natl Acad. Sci. (USA).* 97:13144-13149.

PETERS, H. and BALLING, R. (1999). Teeth: where and how to make them. *Trends Genet.* 15:59-65.

PETERS, H., NEUBSER, A., KRATOCHWIL, K. and BALLING, R. (1998). Pax9-deficient mice lack pharyngeal pouch derivatives and teeth and exhibit craniofacial and limb abnormalities. *Genes Dev.* 12:2735-2747.

QIU, M., BULFORNE, A., GHATTAS, I., MENESES, J.J., CHISTENSEN, L., SHARPE, P.T., PRESLEY, R., PEDERSEN, R.A. and RUBENSTEIN, J.L.R. (1997). Role of Dlx Homeobox Genes in Proximodistal Patterning of the Branchial Arches: Mutations of Dlx-1, Dlx-2, and Dlx-1 and -2 Alter Morphogenesis of Proximal Skeletal and Soft Tissue Structures Derived from the First and Second Arches. *Dev. Biol.* 185:165-184.

RANGANAYAKULU, G., ELLIOTT, D.A., HARVEY, R.P. and OLSON, E.N. (1998). Divergent roles for *NK-2* class homeobox genes in cardiogenesis in flies and mice. *Development.* 125:3037-3048.

REECY, J.M., YAMADA, M., CUMMINGS, K., SOSIC, D., CHEN, C.-Y., EICHELE, G., OLSON, E.N. and SCHWARTZ, R.J. (1997). Chicken Nkx-2.8: A Novel Homeobox Gene Expressed in Early Heart Progenitor Cells and Pharyngeal Pouch-2 and -3 Endoderm. *Dev. Biol.* 188:295-311.

SCHNEIDER, A., BRAND, T., ZWEIGERDT, R. and ARNOLD, H.-H. (2000). Targeted disruption of the Nkx3.1 gene in mice results in morphogenetic defects of minor salivary glands: parallels to glandular duct morphogenesis in prostate. *Mech. Dev.* 95:163-174.

SHIMELD, S.M. and HOLLAND, P.W.H. (2000). Vertebrate innovations. *Proc. Natl Acad. Sci. USA* 97:4449-4452.

SMITH, D.M., NIELSEN, C., TABIN, C.J. and ROBERTS, D.J. (2000). Roles of BMP signalling and Nkx2.5 in patterning at the chick midgut-foregut boundary. *Development.* 127:3671-3681.

SPARROW, D.B., KOTECHA, S., CHENLENG, C., LATINKIC, B., COOPER, B., TOWERS, N., EVANS, S.M. and MOHUN, T.J. (2000). Regulation of *tinman* homologues in *Xenopus* embryos. *Dev. Biol.* 227:65-79.

STANLEY, E., BIBEN, C., ELEFANTY, A., BARNETT, L., KOENTGEN, F., ROBB, L. and HARVEY, R.P. (2002). Efficient Cre-Mediated Deletion of Cardiac Progenitor Cells Conferred by a 3'utr-IRES-Cre Allele of the Homeobox Gene *Nkx2-5. Int. J. Dev. Biol.* this issue.

SU, D.-M., ELLIS, S., NAPIER, A., LEE, K. and MANLEY, N.R. (2001). Hoxa3 and Pax1 Regulate Epithelial Cell Death and Proliferation during Thymus and Parathyroid Organogenesis. *Dev. Biol.* 236:316-329.

TANAKA, M., SCHINKE, M., LIAO, H.-S., YAMASAKI, N. and IZUMO, S. (2001). Nkx2.5 and Nkx2.6, Homologs of *Drosophila* tinman, Are Required for Development of the Pharynx. *Mol. Cell. Biol.* 21:4391-4398.

TANAKA, M., YAMASAKI, N. and IZUMO, S. (2000). Phenotypic characterisation of the murine Nkx2.6 homeobox gene by gene targeting. *Mol. Cell. Biol.* 20:2874-2879.

TRAINOR, P.A. and KRUMLAUF, R. (2001). *Hox* genes, neural crest cells and branchial arch patterning. *Curr. Opin. Cell Biol.* 13:698-705.

TUCKER, A.S., MATTHEWS, K.L. and SHARPE, P.T. (1998). Transformation of Tooth Type Induced by Inhibition of BMP Signalling. *Science.* 282:1136-1138.

WANG, C.-C., BIBEN, C., ROBB, L., NASSIR, F., BARNETT, C., DAVIDSON, N.O., KOENTGEN, F., TARLINTON, D. and HARVEY, R.P. (2000). Homeodomain Factor Nkx2-3 Controls Regional Expression of Leukocyte Homing Coreceptor MAdCAM-1 in Specialised Endothelial Cells of the Viscera. *Dev. Biol.* 224:152-167.

WENDLING, O., DENNEFELD, C., CHAMBON, P. and MARK, M. (2000). Retinoid signaling is essential for patterning the endoderm of the third and fourth pharyngeal arches. *Development.* 127:1553-1562.

YOUNG, J.A. and VAN LENNEP, E.W. (1978). *The Morphology of Salivary Glands.* Academic Press, London.

YUAN, B., LI, C., KIMURA, S., ENGELHARDT, R.T., SMITH, B.R. and MINOO, P. (2000). Inhibition of Distal Lung Morphogenesis in *Nkx2.1(-/-)* Embryos. *Dev. Dynamics.* 217:180-190.