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BASIC AND TRANSLATIONAL—ALIMENTARY TRACT

Proper Development of the Outer Longitudinal Smooth Muscle of the Mouse Pylorus Requires Nkx2-5 and Gata3

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BACKGROUND & AIMS: Infantile hypertrophic pyloric stenosis is a common birth anomaly characterized by obstruction of the pyloric lumen. A genome-wide association study implicated *NKX2-5*, which encodes a transcription factor that is expressed in embryonic heart and pylorus, in the pathogenesis of infantile hypertrophic pyloric stenosis. However, the function of the NKX2-5 in pyloric smooth muscle development has not been examined directly. We investigated the pattern of *Nkx2-5* during the course of murine pyloric sphincter development and examined coexpression of *Nkx2-5* with *Gata3* and *Sox9*—other transcription factors with pyloric-specific mesenchymal expression. We also assessed pyloric sphincter development in mice with disruption of *Nkx2-5* or *Gata3*. **METHODS:** We used immunofluorescence analysis to compare levels of NKX2-5, GATA3, and SOX9 in different regions of smooth muscle cells. Pyloric development was assessed in mice with conditional or germline deletion of *Nkx2-5* or *Gata3*, respectively. **RESULTS:** *Gata3, Nkx2-5*, and *Sox9* are coexpressed in differentiating smooth muscle cells of a distinct fascicle of the pyloric outer longitudinal muscle. Expansion of this fascicle coincides with development of the pyloric sphincter. Disruption of *Nkx2-5* or *Gata3* causes severe hypoplasia of this fascicle and alters pyloric muscle shape. Although expression of *Sox9* requires *Nkx2-5* and *Gata3*, there is no apparent hierarchical relationship between *Nkx2-5* and *Gata3* during pyloric outer longitudinal muscle development. **CONCLUSIONS:** *Nkx2-5* and *Gata3* are independently required for the development of a pyloric outer longitudinal muscle fascicle, which is required for pyloric sphincter morphogenesis in mice. These data indicate that regulatory changes that alter *Nkx2-5* or *Gata3* expression could contribute to pathogenesis of infantile hypertrophic pyloric stenosis.

Keywords: Infantile Hypertrophic Pyloric Stenosis; Primary Duodenogastric Reflux; Sox9; Smooth Muscle Development.

The pyloric sphincter integrates neuronal and hormonal signals to control the movement of food from the stomach to the small intestine.[1] This sphincter is clinically significant in the context of the common human congenital pathology, infantile hypertrophic pyloric stenosis (IHPS), in which both the structure and function of the sphincter are abnormal.[2–4] Infants with IHPS classically present 3 to 6 weeks after birth with projectile vomiting, as well as physical and radiographic findings of gastric outlet obstruction. The etiology of IHPS appears to be complex and can involve both environmental and genetic factors. Changes in musculature, mucosa, extracellular matrix, nerve conduction, and nitric oxide signaling have all been implicated in IHPS pathogenesis.[2–4]

A recent genome-wide association study in humans identified several IHPS susceptibility loci, including the homeodomain transcription factor *NKX2-5*.[5] This is of interest because *Nkx2-5* is expressed in pyloric mesenchyme during embryogenesis in frog, chick, and mouse,[6] although the precise identity of the expressing cells is unknown. Despite its evolutionarily conserved pyloric expression and association with IHPS, the role of *Nkx2-5* in pyloric development has not been examined in vertebrate models, in part because *Nkx2-5* null mice die of cardiac abnormalities at embryonic day (E) 10, well before the pyloric region is fully developed.[7]

Work in the chick model suggests that bone morphogenetic protein (BMP) signaling controls the expression of both *Nkx2-5* and the sex-determining region Y–related, high mobility group box gene *Sox9*.[8–11] Functionally, loss of either *Nkx2-5* or *Sox9* expression in the chick affects the character of the pyloric epithelium but has no effect on the pyloric musculature,[8–11] suggesting that these mesenchymal factors act indirectly to control the expression of an unknown modulator of epithelial phenotype.

In the mouse, direct functional analysis of *Nkx2-5* or *Sox9* at the pylorus has not been reported, however, other genetic models of pyloric sphincter dysmorphogenesis have been described.[12–16] For example, germline deficiency of *Six2*, a homeodomain transcription factor expressed in the posterior stomach, abrogates *Sox9* expression and temporarily

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Abbreviations used in this paper: αSMA, α-smooth muscle actin; BMP, bone morphogenetic protein; E, embryonic day; ICM, inner circular muscle; IHPS, infantile hypertrophic pyloric stenosis; OLM, outer longitudinal muscle; SRF, serum response factor; WT, wild type.

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reduces *Nkx2-5* expression at the pylorus, although this expression is later recovered.[16] Importantly, in *Six2* mutant mice, the pyloric musculature and its corresponding luminal constriction are highly attenuated, indicating that *Six2* and/or one or more of its downstream targets is important for pyloric sphincter development.

Although a role for *Nkx2-5* in the formation of the pyloric sphincter might be inferred from the phenotype of *Six2* null mice, a direct connection between *Nkx2-5* and sphincter muscle development has not been demonstrated in either the mouse or chick models. In fact, although it is clear from previous studies that *Nkx2-5* is expressed in pyloric mesenchyme, we present here the first analysis of its expression at the cellular level during pyloric sphincter development and correlate this expression pattern with development of the sphincter muscles.

We find that NKX2-5 protein is expressed in myofibroblasts and smooth muscle cells of the pylorus. NKX2-5 expression is most robust in a dorsal fascicle of outer longitudinal muscle (OLM) that matures between E14.5 and E16.5. Interestingly, the cells of this OLM fascicle also express SOX9, as well as GATA3, a zinc finger transcription factor that we previously identified as a pylorus-specific gene.[17] After germline deletion of *Gata3* or conditional deletion of *Nkx2-5,* the dorsal pyloric OLM fascicle is hypoplastic, the shape of the inner circular muscle (ICM) is altered, and constriction of the pyloric sphincter is attenuated. Together, these data reveal a distinct transcriptional regulatory cascade that is used for development of the dorsal pyloric OLM; correct development of this fascicle is required to generate the proper morphology of the pyloric sphincter. These findings have implications for the potential role of *NKX2-5* in the pathogenesis of IHPS in humans.

Materials and Methods

Mice

All protocols for mouse experiments were approved by and carried out in accordance with the policies of the University of Michigan University Committee of Use and Care of Animals and Unit for Laboratory Animal Medicine. C57BL/6J inbred (wild type [WT]) mice were obtained from Charles River Laboratories (Wilmington, MA). The generation of *Gata3*$^{lacZ/+}$, *Nkx2-5*$^{lacZ/+}$, and *CAGGCre-ER*^{TM} mice has been described previously.[18–20]

Gata3 null embryos were generated via *Gata3*$^{lacZ/+}$ intercrosses. To escape early embryonic lethality, *Gata3* null embryos were pharmacologically rescued in utero by treating timed-pregnant dams with α- and β-adrenergic agonists, as described previously.[21,22] The rescue solution was administered once daily via a water bottle, beginning at E7.5, and all other drinking water was withheld. Rescue solution was prepared fresh, as follows: 15 mg each of isoproterenol (I-5627; Sigma-Aldrich, St Louis, MO) and phenylephrine (P-6126; Sigma-Aldrich) was added to 50 mL of water and supplemented with 100 mg ascorbic acid and 2 g sucrose.

Nkx2-5$^{flox/+}$ mice were generated by targeted homologous recombination in embryonic stem cells, as described previously.[23,24] The targeting construct was created by cloning a neomycin resistance cassette, flanked by FLP recognition target sites, into intron 1 of *Nkx2-5*; loxP sites were then cloned upstream of the neomycin resistance cassette and downstream of the homeodomain-containing exon 2 (Supplementary Figure 1*B*). The neomycin resistance cassette was excised via FLP-mediated recombination, resulting in loxP sites flanking exon 2 (Supplementary Figure 1*C*). *Nkx2-5*$^{flox/+}$ mice were crossed to *CAGGCre-ER*^{TM} (004682; The Jackson Laboratory, Bar Harbor, ME) and bred to homozygosity for the conditional allele (*CAGGCre-ER*^{TM};*Nkx2-5*$^{flox/flox}$). Inactivation of *Nkx2-5* in timed-pregnant dams was accomplished via intraperitoneal injections of tamoxifen (T5648; Sigma-Aldrich), as described previously.[19] Briefly, pregnant dams were injected with 150 μL tamoxifen-corn oil solution (20 mg tamoxifen per mL of corn oil) once daily for up to 2 days before embryo harvest.

Protocols for genotyping, bromodeoxyuridine labeling, whole-mount X-gal staining, routine tissue fixation and processing, and immunostaining and quantitation are provided in the Supplementary Materials.

Results

Development of Pyloric Muscular Components

Despite the important function of the pyloric sphincter, development of its smooth muscle components has not been assessed at the cellular level. We therefore examined sectioned pyloric tissue using H&E staining and immunofluorescence for α-smooth muscle actin (αSMA), a marker of differentiated smooth muscle cells and myofibroblasts. At E14.5, the ICM at the pylorus is contiguous with that of the surrounding stomach and intestine and strongly expresses αSMA (Figure 1*A* and *B*). In contrast, the nascent OLM contains a thin layer of weakly αSMA-positive cells (Figure 1*Bv* and *vi*, *asterisk*). These cells are not yet tightly organized into muscular bundles. Dorsally, these cells bridge directly to and intermingle with a prominent collection of αSMA-negative pancreatic mesenchymal cells (Figure 1*Aii*).

By E16.5, the pyloric OLM is compacted and robustly expresses αSMA, indicative of smooth muscle differentiation (Figure 1*C* and *D*). Dorsally, a thickened fascicle of OLM appears to displace the ICM internally (Figure 1*Dv* and *vi*, *asterisk*), thereby narrowing the pyloric lumen to generate the characteristic constriction of the mature pyloric sphincter.

Nkx2-5 and Gata3 Are Expressed in Similar Domains at the Pylorus

Previous studies have shown that as early as E9.5, a mesenchymal *Nkx2-5* expression domain surrounds the nascent distal stomach and proximal duodenal endoderm.[25] By E12.5, some cells within this mesenchymal domain have migrated anteriorly along the dorsal left side of the stomach to give rise to the spleen, while others remain at the pylorus.[25] Because the late embryonic expression pattern of *Nkx2-5* at the pylorus has not been carefully described, we examined whole-mount X-gal staining of dissected tissue