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

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Abstract

Background & Aims—Infantile hypertrophic pyloric stenosis (IHPS) is a common birth anomaly characterized by obstruction of the pyloric lumen. A genome-wide association study

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implicated *NKX2-5*, which encodes a transcription factor that is expressed in embryonic heart and pylorus, in the pathogenesis of IHPS. However, the function of the NKX2-5 in pyloric smooth muscle development has not been directly examined. We investigated the pattern of Nkx2-5 during the course of murine pyloric sphincter development and examined co-expression 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* were co-expressed in differentiating smooth muscle cells of a distinct fascicle of the pyloric outer longitudinal muscle (OLM). Expansion of this fascicle coincided with development of the pyloric sphincter. Disruption of *Nkx2-5* or *Gata3* caused severe hypoplasia of this fascicle and alters pyloric muscle shape. Although expression of *Sox9* required *Nkx2-5* and *Gata3*, there was no apparent hierarchical relationship between *Nkx2-5* and *Gata3* during pyloric OLM development.

Conclusions—*Nkx2-5* and *Gata3* are independently required for the development of a pyloric OLM fascicle, which 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 IHPS.

Keywords

Infantile hypertrophic pyloric stenosis; primary duodenogastric reflux; Sox9; smooth muscle development

Introduction

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 three to six 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 may 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 (GWAS) 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 E10[7], well before the pyloric region is fully developed.

Work in the chick model suggests that BMP signaling controls the expression of both *Nkx2-5* and the SRY-related, HMG-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 reduces *Nkx2-5* expression at the pylorus, though 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.

Though 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, while 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 embryonic days (E) 14.5 and 16.5. 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*™ 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 previously described[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 (Sigma-Aldrich, St. Louis, MO, I-5627) and phenylephrine (Sigma-Aldrich, P-6126) was added to 50 mL of water and supplemented with 100 mg of ascorbic acid and 2 g of 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 (FRT) 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 (Supplemental Figure 1B). The neomycin resistance cassette was excised via FLP-mediated FRT site recombination,

resulting in loxP sites flanking exon 2 (Supplemental Figure 1C). *Nkx2-5*$^{flox/+}$ mice were crossed to *CAGGCre-ER*™ (The Jackson Laboratory, Bar Harbor, ME, 004682) and bred to homozygosity for the conditional allele (*CAGGCre-ER*™;*Nkx2-5*$^{flox/flox}$). Inactivation of *Nkx2-5* in timed-pregnant dams was accomplished via intraperitoneal injections of tamoxifen (Sigma-Aldrich, T5648), as described previously[19]. Briefly, pregnant dams were injected with 150 μL of tamoxifen-corn oil solution (20 mg tamoxifen per mL of corn oil) once daily for up to two days prior to embryo harvest.

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

Results

Development of pyloric muscular components

Despite the important function of 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 alpha 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 1A,B). In contrast, the nascent OLM contains a thin layer of weakly αSMA positive cells (Figure 1Bv,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 1Aii, PM).

By E16.5, the pyloric OLM is compacted and robustly expresses αSMA, indicative of smooth muscle differentiation (Figure 1C,D). Dorsally, a thickened fascicle of OLM appears to displace the ICM internally (Figure 1Dv,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]. Since 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 from *Nkx2-5*$^{lacZ/+}$ embryos. At E14.5, *Nkx2-5*-expressing cells encircle the pylorus, and staining on the dorsal side of the pylorus extends into adjoining pancreatic mesenchyme (Figure 2Ai). Bilateral cellular cords, emanating from the pylorus and reaching across the lesser curvature of the antrum, also express *Nkx2-5* (Figure 2Ai, black arrowheads); these structures likely correspond to the previously described gastric ligaments[26]. Continuity between the X-gal positive pyloric band and gastric ligaments is obvious ventrally (Figure 2Aii). Between E14.5 and E18.5, the gastric ligaments lengthen to reach the gastroesophageal junction, but the *Nkx2-5* expression pattern is otherwise unchanged (Figure 2Aiii).

In a previous study, we found that *Gata3* exhibits a pyloric-specific expression domain in the mesenchyme, similar to that of *Nkx2-5*[17]. To further characterize *Gata3* expression at the pylorus, we examined dissected whole mount X-gal stained tissue from *Gata3*$^{lacZ/+}$ embryos. Similar to *Nkx2-5*, *Gata3* is expressed in a discrete band at the pylorus, as well as in the gastric ligaments (Figure 2Bi, black arrowheads). Notably, the pyloric *Gata3*

expression domain is narrower than *Nkx2-5*, and on the lesser curvature (ventral) side, there is a small gap in the *Gata3* expression domain (Figure 2Bii, red arrowhead). The nature of this gap is further explored below.

Co-expression of pyloric transcription factors during pyloric sphincter maturation

Self *et al*. previously showed that, between E12.5 and E14.5, *Nkx2-5* and *Sox9* are expressed in similar domains at the murine pylorus[16]. This is important since both of these genes have been implicated in pyloric development[8–11]. However, we were interested to: a) determine whether *Nkx2-5* and *Sox9* are expressed in the same cells; b) compare these domains with the expression pattern of *Gata3*; and, c) examine expression of these genes during establishment of the complete pyloric musculature (from E14.5 to E16.5).

The results of this expression analysis reveal distinct pyloric cell populations that are single, double, or triple positive for NKX2-5, SOX9, and/or GATA3, suggesting that a complex developmental circuitry directs pyloric development. At both E14.5 and E16.5, the expression domains of GATA3 and NKX2-5 are exclusively mesenchymal, while SOX9 is expressed in both epithelium and mesenchyme, as previously noted (Figure 3)[16]. A line of enteric neurons that are strongly positive for SOX9 and the neuronal marker peripherin, but negative for GATA3 and NKX2-5 (Supplemental Figure 2), separates the OLM and ICM domains (white lines in Figure 3). At E14.5, cells that are exclusively NKX2-5 positive are detected in the sub-epithelial mesenchyme and in the ICM (Figure 3Aviii, yellow arrowheads), while cells that express only GATA3 are located in the outermost dorsal pylorus, where it mixes with pancreatic mesenchyme (Figure 3Aix, white arrowheads). Some of these GATA3 positive cells are squamous in morphology and may be part of the developing serosa (Supplemental Figure 3).

Cells that are triple positive for NKX2-5, GATA3, and SOX9 are visible at E14.5 as a loose cluster of cells located primarily within the nascent OLM territory on the dorsal side (Figure 3Av,x, asterisk). Comparison with Figure 1 indicates that some of these cells are weakly αSMA positive (Figure 1Bvi, asterisk). Cross sections of the E14.5 pylorus confirm the low expression of αSMA in the OLM, which is marked by expression of both GATA3 and NKX2-5 (Supplemental Figure 4). Notably, analysis of multiple cross sections confirms that the OLM is absent on the ventral side of the pylorus between the nascent gastric ligaments, accounting for the apparent gap in *Gata3* expression seen in X-gal stained whole mount tissue above (Figure 2Bii).

At E16.5, cells of the OLM strongly express αSMA (Figure 1Dvi, asterisk). Additionally, OLM cells continue to express SOX9, NKX2-5 and GATA3 (Figure 3Bv,x, asterisk). Thus, cells that co-express all three transcription factors appear to contribute primarily to the OLM.

Nkx2-5 is required for development and maintenance of the pyloric OLM

The expression pattern of *Nkx2-5* suggests a potential role for this transcription factor in pyloric development. However, germline deficiency of *Nkx2-5* results in embryonic lethality by E10[7], precluding analysis of *Nkx2-5* function in late embryogenesis. Thus, we paired a conditional *Nkx2-5* allele (*Nkx2-5*$^{flox/flox}$) with a tamoxifen-inducible Cre recombinase transgene driven from a ubiquitously expressed transgenic promoter (*CAGGCre-ER*™)[19]. Two cohorts of pregnant dams were treated with once-daily tamoxifen injections, beginning at E14.5 or E16.5; each cohort was sacrificed two days later for analysis.

Deletion of *Nkx2-5* beginning at E14.5 leads to nearly complete absence of the αSMA positive pyloric OLM smooth muscle at E16.5 (Figure 4Av,vi, asterisks), suggesting that

Nkx2-5 is necessary for the maturation of the OLM. Interestingly, *Nkx-5* deletion beginning at E16.5, when the OLM is already formed and strongly expresses αSMA (Figure 1Dv,vi), significantly reduces OLM smooth muscle by E18.5 (Figure 4Bv,vi, asterisks and Supplemental Figure 5C). Remaining cells are loosely organized and weakly rather than strongly αSMA positive, suggesting that *Nkx2-5* function is continuously required for the maintenance of differentiated smooth muscle cells within this fascicle.

Increased apoptosis and reduced proliferation in pyloric OLM after loss of Nkx2-5

Our data show that loss of *Nkx2-5* beginning at E14.5 prevents the maturation of the pyloric OLM fascicle, and deletion of this gene after the fascicle has formed at E16.5 leads to its regression. Reduced smooth muscle cell proliferation and/or increased apoptosis are potential mechanisms underlying these OLM responses. To examine proliferative changes, timed-pregnant dams were injected with BrdU two hours prior to sacrifice. In WT animals, BrdU positive cells were found scattered throughout the ICM and OLM regions at E14.5 and E16.5 (Supplemental Figure 6A). Conditional deletion of *Nkx2-5* beginning at E14.5 was associated with a 25% reduction in the proportion of proliferative cells in the OLM fascicle at E16.5 (19.6% in WT versus 14.8% after *Nkx2-5* deletion, $P < 0.05$) (Supplemental Figure 6B,Ci). Though *Nkx2-5* is also expressed in the ICM, no significant change in proliferative activity was detectable in that domain (Supplemental Figure 6Cii).

To assess the impact on apoptosis, we examined caspase 3 (CASP3) expression 24 and 48 hours after deletion of *Nkx2-5*. At 48 hours post-deletion, the OLM fascicle was largely absent, and the remaining cells were not CASP3 positive (data not shown). At 24 hours post-deletion, though the total number of cells (as well as the number of NKX2-5 positive cells) had already decreased, the proportion of CASP3 positive cells within the OLM had nearly doubled (2.6% in WT versus 4.8% after *Nkx2-5* deletion, $P < 0.05$) (Supplemental Figure 7). We conclude that deletion of *Nkx2-5* results in decreased proliferation, increased apoptotic activity and rapid loss of the dorsal pyloric OLM fascicle. Interestingly, though the ICM also expresses *Nkx2-5*, proliferation and apoptosis within this domain does not appear to be altered, indicating that a distinct molecular network directs the maturation of this muscle cell population.

Gata3 is necessary for the formation of the pyloric OLM fascicle

Since cells of the dorsal pyloric OLM express both *Gata3* and *Nkx2-5* (Figure 3), we next examined the consequences of germline *Gata3* loss on the development of this fascicle. Though germline *Gata3* deficiency results in early embryonic lethality, *Gata3* null embryos can be pharmacologically rescued by catecholamine administration *in utero* and will survive until birth, permitting the analysis of *Gata3* function in later fetal development[21, 22]. We found no obvious pyloric abnormalities in *Gata3* null animals at E14.5 (data not shown). However, by E16.5, *Gata3* deficiency results in nearly complete absence of the OLM fascicle (Figure 5Aiv–vi, asterisks and Supplemental Figure 5B), a phenotype highly similar to that seen after *Nkx2-5* conditional deletion.

Loss of Gata3 or Nkx2-5 alters ICM morphogenesis and pyloric sphincter constriction

As shown in Figures 4A and 5A, conditional *Nkx2-5* deletion or germline *Gata3* deficiency leads to loss of the dorsal OLM fascicle. In both cases, at E16.5, the shape of the dorsal ICM is clearly altered, as depicted in the tracings shown in Figure 5B. Since *Gata3* is not widely expressed in the ICM (Figure 3), the shape change detected in this mutant model must be secondary to the loss of the OLM. In addition, at E18.5, the pyloric sphincter constriction is attenuated in both genetic deficiency models; compared to WT (95% CI = 231–266 μm), the constriction is 40% wider in *Gata3* null animals (95% CI = 267–450 μm, $P < 0.05$) and 22% wider after conditional *Nkx2-5* deletion (95% CI = 228–379 μm, $P = 0.13$) (Figure 5C).

A regulatory hierarchy of pyloric transcription factors

We next examined the expression of NKX2-5, GATA3 and SOX9 in E16.5 pyloric smooth muscle after conditional *Nkx2-5* deletion or in *Gata3* null mice. Deletion of *Nkx2-5* starting at E14.5 effectively ablates NKX2-5 expression in the majority of cells at the E16.5 pylorus (Figure 6Biii). While SOX9 staining is absent in smooth muscle cells (Figure 6Bii), GATA3 expression persists in NKX2-5 negative cells of the OLM remnant (Figure 6Biv), suggesting that *Nkx2-5* is required for *Sox9* but not *Gata3* expression in this domain.

In *Gata3* null mice, both GATA3 (Figure 6Civ) and SOX9 (Figure 6Cii) are absent in smooth muscle cells, but NKX2-5 expression is apparent in cells of the reduced OLM territory at E16.5 (Figure 6Ciii). Persistence of NKX2-5 expression was additionally confirmed at E14.5 (Supplemental Figure 8). Thus, *Gata3* is required for *Sox9* expression, but not *Nkx2-5* expression, in the dorsal pyloric OLM. Taken together, these data reveal a pyloric transcriptional hierarchy in which *Nkx2-5* and *Gata3* are independently required to drive *Sox9* expression and, consequently, smooth muscle development within the dorsal OLM fascicle.

Loss of Nkx2-5 or Gata3 does not affect the epithelial pyloric border

In the chick, perturbation of mesenchymal NKX2-5 activity changes the character of the pyloric epithelium, suggesting that signaling molecules driven by NKX2-5 are responsible for dictating epithelial phenotype[10, 11]. In the mouse, the distinct characters of the stomach and duodenal epithelia are established between E14.5 and E16.5[17, 27] , a time coincident with the development of the OLM fascicle (Figure 1). Therefore, we assessed the integrity of the epithelial stomach-intestinal (pyloric) border at E18.5 after conditional *Nkx2-5* deletion and in *Gata3* null animals by examining the expression of CDX2, an intestine-specific epithelial marker. In both models, the position of the epithelial pyloric border was similar to WT (Supplemental Figure 9). Thus, in the mouse, neither *Gata3* nor *Nkx2-5* is required for positioning of the epithelial pyloric border.

Discussion

Here, we provide a detailed analysis of normal pyloric muscle development at the cellular level, revealing an unexpectedly complex expression pattern of three critical transcription factors in these muscles. We provide evidence for a redundant regulatory mechanism that controls the development of a specific dorsal OLM fascicle at the pylorus. This fascicle is first detectable as a population of loosely organized, weakly αSMA positive smooth muscle cells at E14.5; it organizes considerably over the next two days, becoming highly αSMA positive and displacing the ICM to generate a distinct pyloric sphincter constriction by E16.5. Cells within this dorsal OLM fascicle express NKX2-5, GATA3, and SOX9. Deletion of either *Nkx2-5* or *Gata3* abrogates development of this OLM fascicle, results in loss of SOX9 expression, and alters pyloric muscular shape. While both *Nkx2-5* and *Gata3* are also expressed in other cells at the pylorus, loss of either factor appears to affect only the phenotype of triple positive cells (NKX2-5, GATA3, and SOX9) within the dorsal OLM fascicle.

This is the first study to characterize *Gata3* pyloric expression at the cellular level and to establish a role for *Gata3* in pyloric development. We find that GATA3 is expressed in cells on the serosal surface (Supplemental Figure 3) and in cells that intermix with pancreatic mesenchyme (Figure 3); neither of these cell types co-expresses NKX2-5 or SOX9, and their functional role remains undetermined since no obvious phenotype was detected in serosa or pancreas in *Gata3* deficient mice. In contrast, the effect of *Gata3* loss on the pyloric OLM fascicle is dramatic and directly mirrors the effects of *Nkx2-5* loss. Despite this

similarity in phenotype, we found no clear evidence of a transcriptional relationship between *Gata3* and *Nkx2-5*. Further supporting the independent regulation of these factors, conditional overexpression of *Wnt9b*, or activation of a stabilized form of beta-catenin in the distal stomach and pylorus affects *Nkx2-5* and *Gata3* expression oppositely: the *Nkx2-5* domain is expanded, while expression of *Gata3* is abolished[12].

Interestingly, both *Nkx2-5* and *Gata3* are required for pyloric sphincter morphogenesis and both directly or indirectly regulate *Sox9* expression. Three other transcription factors, *Barx1*, *Bapx1*, and *Six2*, are also important for pyloric sphincter development and *Sox9* expression[13–16]. All three factors are expressed in distal stomach as well as pylorus. It has been proposed that *Bapx1* functions downstream of *Barx1*, since its expression is lost in *Barx1* mutant mice[14]. Interestingly, loss of *Bapx1* does not affect *Nkx2-5* expression, but gene expression microarrays demonstrate decreased *Sox9* in *Bapx1* null animals[14, 15]. Thus, *Bapx1* may regulate *Sox9* expression directly or indirectly via *Gata3*. Loss of *Six2* also results in attenuation of the pyloric sphincter constriction and is associated with transient loss of *Nkx2-5* and complete loss of SOX9 expression[16]. Thus, all of these models converge on *Sox9* and given its expression in the OLM (Figure 3), it will be important to directly determine whether *Sox9* is required for development of this OLM fascicle and establishment of pyloric sphincter constriction. Testing this possibility will require a transgenic *Cre* driver that is active in the pyloric mesenchyme but not the epithelium, where *Sox9* plays a critical role in establishing the stem cell zone[28, 29].

Both *Nkx2-5* and *Gata3* are important for proper differentiation of the OLM fascicle, since in their absence, cells are disorganized and only weakly αSMA positive. Even very late deletion of *Nkx2-5* (after the OLM has fully developed) results in an OLM fascicle that is reduced in size, organization, and differentiation. Studies in the chick suggested that a zone of *Bmp4* exclusion is important for initial positioning of the *Nkx2-5* and *Sox9* expression domains at the pylorus[8–11] , and ectopic expression of *Bmp4* in this zone compromised smooth muscle differentiation[9]. Once positioned, if *Nkx2-5* and/or *Gata3* function to suppress *Bmp4*, ectopic BMP signaling might be responsible for the increased apoptosis and reduced proliferation and differentiation seen after deletion of these factors. However, by Smad1/5/8 staining, we were unable to detect a zone of low BMP pathway activity at the E16.5 WT pylorus and saw no change in staining after loss of *Nxk2-5* or *Gata3* (data not shown). In accord with this finding, loss of *Gata3* (due to overexpression of *Wnt9b* at the pylorus) compromises pyloric sphincter formation but does not alter *Bmp4* expression[12]. Thus, the importance of BMP signaling in later pyloric development remains to be clarified.

Co-regulation of muscle differentiation genes by NKX and GATA family members has been seen in other systems: NKX3-2 and GATA6 cooperatively activate smooth muscle genes (e.g., *Itga1*, *SM22*α, and *Cald1*), while NKX2-5 and GATA4 co-activate cardiac muscle genes (e.g., *Anf*)[30]. In both cases, co-regulation also involves serum response factor (SRF), an important regulator of muscle gene expression in differentiating card iac and smooth muscle. These three proteins together (SRF/GATA/NKX) synergistically activate target gene expression at levels 5–10 fold greater than SRF/GATA or SRF/NKX alone[30]. Thus, it is possible that loss of either *Nkx2-5* or *Gata3* disrupts ternary complexes of GATA3, NKX2-5, and SRF that are required for the expression of genes involved in the differentiation and/or maintenance of pyloric smooth muscle. This remains to be directly tested.

Failure to establish or maintain the proper pyloric musculature, as seen in both the conditional *Nkx2-5* deletion and *Gata3* null models examined here, could potentially underlie some cases of primary duodenogastric reflux, a rare and poorly understood condition involving excessive reflux of bile acids from the duodenum to the stomach[31].

IHPS, in contrast, presents as pyloric obstruction. Given that recent GWAS data link nucleotide polymorphisms near the *NKX2-5* gene locus to IHPS[5], our results would predict that some forms of IHPS may result from overexpression of *NKX2-5* at the pylorus. For example, a change in an intergenic regulatory element could readily account for increased pyloric *NKX2-5* expression. Indeed, an upstream enhancer that drives pyloric *Nkx2-5* expression in mice has already been described[24], and it will be interesting to examine the homologous region in patients with IHPS. Additionally, our data suggest that the search for linked polymorphisms in IHPS should be expanded to include *GATA3* and, potentially, *SOX9*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

αSMA	alpha smooth muscle actin
BrdU	bromodeoxyuridine
BMP	bone morphogenetic protein
CASP3	caspase 3
DGR	duodenogastric reflux
E	embryonic day
FRT	FLP recognition target
GWAS	genome-wide association study
H&E	hematoxylin and eosin
HMG	high mobility group
ICM	inner circular muscle
IHPS	infantile hypertrophic pyloric stenosis
OLM	outer longitudinal muscle

SRF	serum response factor
SRY	sex-determining region Y
WT	wild type

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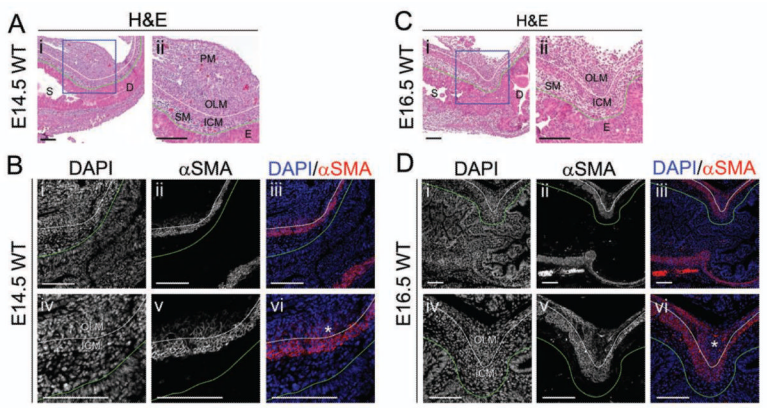

Figure 1. Development of the pylorus between E14.5 and E16.5 involves differentiation of the dorsal OLM and changes in ICM shape

H&E staining of WT pylorus at (A) E14.5 or (C) E16.5; the dorsal pylorus, within the boxed region in (i) is enlarged in (ii). Immunofluorescence of the dorsal WT pylorus at (B) E14.5 or (D) E16.5: (i,iv) DAPI; (ii,v) αSMA; or (iii,vi) merged. (A–B) Though the ICM is well-differentiated and strongly αSMA positive at E14.5, cells of the OLM stain weakly for αSMA (asterisk in Bvi). (C–D) Expansion and differentiation of the OLM (asterisk in Dvi) is associated with an increase in αSMA expression and inward displacement of the ICM, resulting in pyloric sphincter constriction. For all images, stomach (S) is left; duodenum (D) is right; and, dorsal is top. Green lines mark the epithelial basement membrane, and white lines separate ICM and OLM. E = epithelium; SM = sub-epithelial mesenchyme; and, PM = pancreatic mesenchyme. Scale bars represent 100 μm.

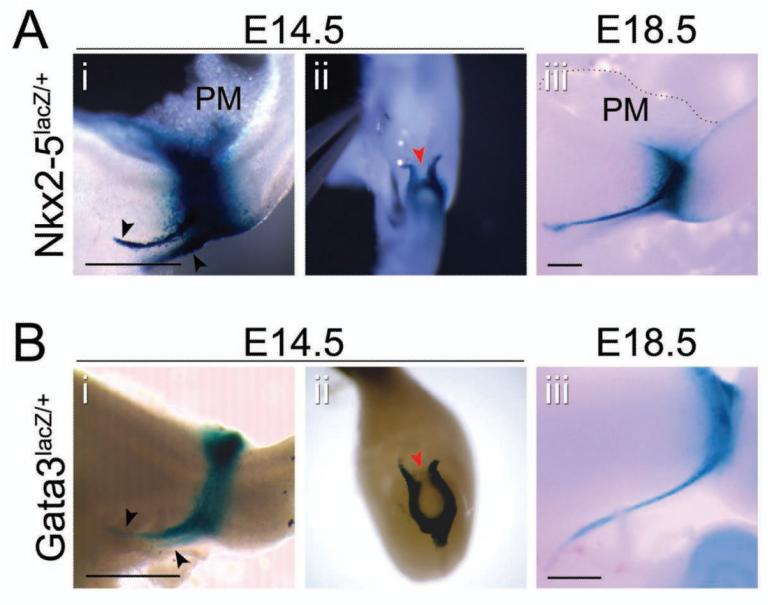

Figure 2. Gata3 and Nkx2-5 are expressed in similar domains at the pylorus

Whole mount X-gal staining of pylorus from (A) *Nkx2-5*$^{lacZ/+}$ or (B) *Gata3*$^{lacZ/+}$ mice at (i–ii) E14.5 and (iii) E18.5. (i,iii) Lateral view: stomach is left; duodenum is right; dorsal is top. (ii) Ventral view: stomach is top; duodenum is bottom. (Ai,Bi) *Nkx2-5* and *Gata3* have similar expression patterns, with extension dorsally into the pancreatic mesenchyme (PM in Aii) and ventrally into the gastric ligaments (black arrowheads in Ai and Bi), however, the width of the *Gata3* expression domain is narrower than *Nkx2-5*. (Aii,Bii) *Nkx2-5* expression is completely circumferential (red arrowhead in Aii), while *Gata3* expression is discontinuous ventrally (red arrowhead in Bii). (Aiii,Biii) By E18.5, the gastric ligaments have lengthened to reach the esophagus (pancreatic mesenchyme outlined by dashed line in Aiii). Scale bars = 100 μm.

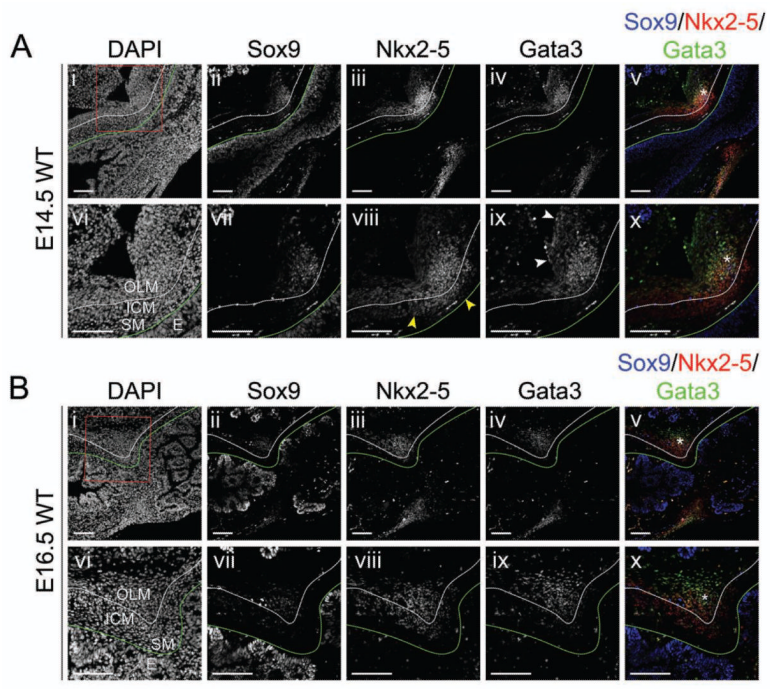

Figure 3. NKX2-5, GATA3, and SOX9 are co-expressed in dorsal pyloric OLM

Immunofluorescence of WT pylorus at (A) E14.5 or (B) E16.5 : (i,vi) DAPI; (ii,vii) SOX9; (iii,viii) NKX2-5; (iv,ix) GATA3; or (v,x) merged. (A–B) NKX2-5 single positive cells are found in the sub-epithelial mesenchyme and ICM (yellow arrowheads in Aviii), while GATA3 single positive cells are present in the pancreatic mesenchyme (white arrowheads in Aix). NKX2-5, GATA3, and SOX9 are co-expressed in the dorsal OLM (asterisks in Av,x and Bv,x). Stomach is left; duodenum is right; and, dorsal is top. Green lines mark the epithelial basement membrane, and white lines separate ICM and OLM. SM = sub-epithelial mesenchyme. Scale bars = 100 μm.

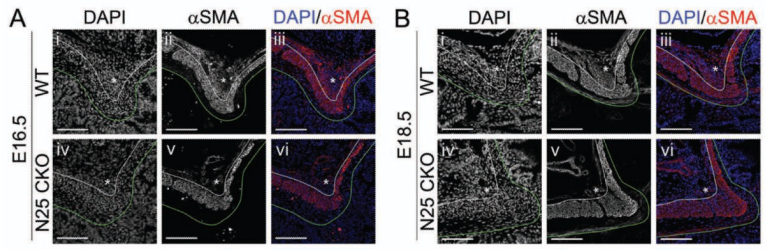

Figure 4. Nkx2-5 is required for the development and maintenance of the dorsal pyloric OLM

Immunofluorescence of (i–iii) *Nkx2-5*$^{flox/flox}$ (WT) or (iv–vi) *CAGGCre-ER*™;*Nkx2-5*$^{flox/flox}$ (N25 CKO) pylorus (after two days of intraperitoneal tamoxifen injections) harvested at (A) E16.5 or (B) E18.5: (i,iv) DAPI; (ii,v) αSMA; or (iii,vi) merged. (A) In N25 CKO mice at E16.5, cell mass and αSMA expression is reduced in the dorsal OLM (asterisks). (B) At E18.5, the dorsal OLM in WT mice has expanded further and shows increased αSMA expression (Bi–iii). Deletion of *Nkx2-5* beginning at E16.5, when the dorsal OLM is already αSMA positive (Aii–iii), leads to its regression (Biv–vi). Stomach is left; duodenum is right; and, dorsal is top. Green lines mark the epithelial basement membrane, and white lines separate ICM and OLM. Asterisk = dorsal pyloric OLM. Scale bars = 100 μm.

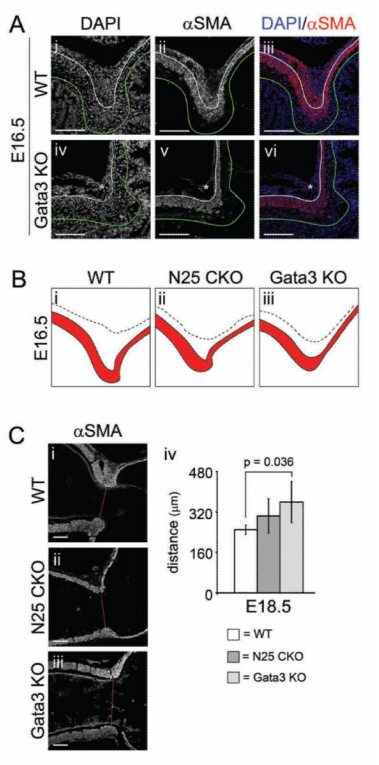

Figure 5. Gata3 is required for formation of the dorsal pyloric OLM; absence of Gata3 or loss of Nkx2-5 alters ICM shape and pyloric sphincter constriction

(A) Immunofluorescence of (i–iii) WT or (iv–vi) *Gata3*$^{lacZ/lacZ}$ (Gata3 KO) pylorus at E16.5: (i,iv) DAPI; (ii,v) αSMA; or (iii,vi) merged. Germline deficiency of *Gata3* results in nearly complete absence of αSMA positive cells in the dorsal pyloric OLM (asterisks in Aiv–vi). (B) Tracings of the pyloric ICM (red) and OLM (white area defined by the dotted line) in WT, *CAGGCre-ER*™;*Nkx2-5*$^{flox/flox}$ (N25 CKO), and Gata3 KO mice at E16.5. Subtle but reproducible changes occur in the shape of the ICM in N25 CKO and Gata3 KO mice. (C) αSMA immunofluorescence of (i) WT, (ii) N25 CKO, or (iii) Gata3 KO pylorus at E18.5. Compared to WT, the pyloric sphincter constriction is somewhat reduced (wider) in N25 CKO animals and significantly attenuated in Gata3 KO (sphincter constriction measurements are shown in Civ). Stomach is left; duodenum is right; and, dorsal is top. Green lines mark the epithelial basement membrane, and white lines separate ICM and OLM. Red lines in (Ci–iii) denote width of pyloric sphincter constriction. Asterisk = dorsal pyloric OLM. Scale bars = 100 μm. Error bars represent one standard deviation from the mean.

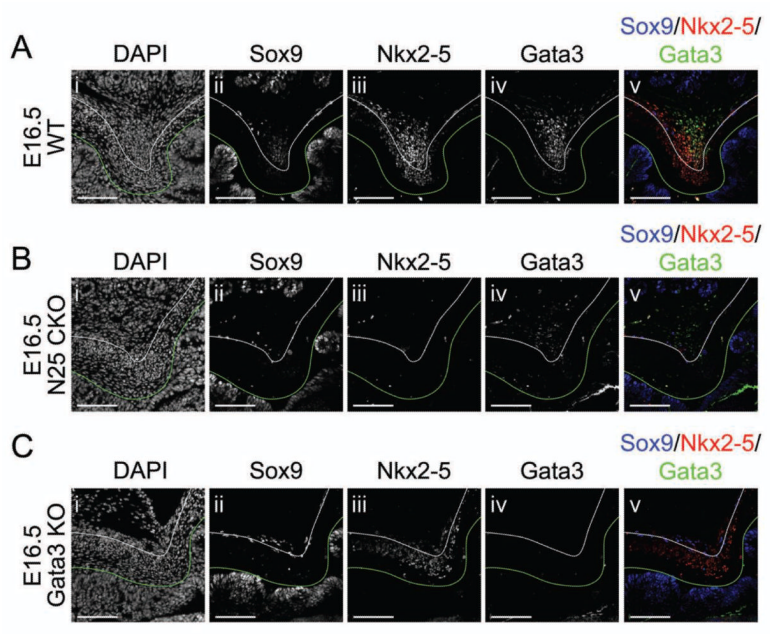

Figure 6. Regulatory hierarchy among Nkx2-5, Gata3 and Sox9 in the dorsal pyloric OLM

Immunofluorescence of (A) WT, (B) *CAGGCre-ER™;Nkx2-5$^{flox/flox}$* (N25 CKO), or (C)) *Gata3$^{lacZ/lacZ}$* (Gata3 KO) pylorus at E16.5: (i) DAPI; (ii) SOX9; (iii) NKX2-5; (iv) GATA3; or (v) merged. (A–C) Conditional loss of *Nkx2-5* effectively eliminates dorsal OLM NKX2-5 expression (Biii), with concomitant loss of SOX9 expression (Bii), but does not abrogate GATA3 expression (Biv). Germline deficiency of *Gata3* is associated with the complete absence of dorsal OLM SOX9 expression (Cii), but NKX2-5 is expressed in rare remaining dorsal OLM cells (Ciii). Stomach is left; duodenum is right; and, dorsal is top. Green lines mark the epithelial basement membrane, and white lines separate ICM and OLM. Scale bars = 100 μm.