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Expression pattern of the homeotic gene \textit{Bapx1} during early chick gastrointestinal tract development

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**A R T I C L E  I N F O**

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**A B S T R A C T**

Regulation of the Bone Morphogenetic Protein (BMP) signaling pathway is essential for the normal development of vertebrate gastrointestinal (GI) tract, but also for the differentiation of the digestive mesenchymal layer into smooth muscles and submucosal layers. Different studies demonstrated that \textit{Bapx1} (for bagpipe homeobox homolog 1) negatively regulates the BMP pathway, but its precise expression pattern during the development and the differentiation of the GI tract mesenchyme actually remains to be examined. Here, we present the spatio-temporal expression profile of \textit{Bapx1} in the chick GI tract. We show that \textit{Bapx1} is first expressed in the undifferentiated mesenchyme of the gizzard and the colon. After the differentiation of the digestive mesenchyme, we found \textit{Bapx1} strongly expressed in the gizzard smooth muscle and in the submucosa layer of the colon. This expression pattern provides new insights into the roles of \textit{Bapx1} during the regionalization of the GI tract and the differentiation of the digestive mesenchyme of the colon and the stomach.

The gastrointestinal (GI) tract is a remarkably complex, three dimensional, specialized and vital organ system derived from a simple tubular structure. The vertebrate GI tract includes the luminal digestive system of the esophagus, stomach, intestines, and colon (which we will designate as "gut") and the GI tract derivatives—thyroid, lungs, liver, and pancreas (Crapin-Botton, 2005; Roberts, 2000; de Santa Barbara et al., 2002). The gut is composed of the three germ layers—mesoderm (which forms the smooth muscle layer), endoderm (which forms the epithelial lining), and ectoderm (which includes the enteric nervous system). Originally, the gut develops from two meso-endodermal invaginations at the anterior (anterior intestinal portal, AIP) and posterior (caudal intestinal portal, CIP) end of the embryo, which elongate and fuse to form a straight tube. This primitive gut tube is initially patterned into three broad domains along its anterior-posterior (AP) axis: the fore-, mid- and hindgut. As they develop, each region of the gut acquires mesodermal and endodermal morphologies, which can be easily discerned by gross and microscopic examination. These tissues differentiate in a regionally specific pattern of differentiation along the AP axis, designating the pharynx, esophagus, and stomach (the foregut), small intestines (the midgut), and large intestines (hindgut). In addition to this AP axis differentiation, the digestive mesenchyme differentiates along the radial axis and gives rise to the submucosa and smooth muscle layers (Faure and de Santa Barbara, 2011).

Candidate factors for GI mesoderm regionalization include nuclear transcription factors (as Hox and Nkx factors) and secreted factors (such as Hedgehog ligands) (de Santa Barbara and Roberts, 2002; Smith et al., 2000; Ramalho-Santos et al., 2000). Bone morphogenetic proteins (BMP) are secreted signaling molecules that belong to the Transforming Growth Factor \(\beta\) (TGF\(\beta\)) superfamily. BMP ligands were initially identified as key regulators of bone formation (Urist et al., 1979), but subsequent analyses have demonstrated that these ligands regulate a spectrum of developmental processes throughout embryogenesis and organogenesis (reviewed in Hogan (1996); de Santa Barbara et al., 2003). Early during GI development, \textit{Bmp4} is expressed in the mesenchyme of the entire gut exclusive of the gizzard (Roberts et al., 1998). When misexpressed in the gizzard, \textit{Bmp4} induces an upregulation of the pyloric sphincter marker Nkx2.5 in the posterior gizzard mesoderm (Smith and Tabin, 1999; Smith et al., 2000). These experiments indicate that a low level of BMP signaling activity is a prerequisite for the correct regionalization of the stomach. Out of the candidate genes for regulating BMP signaling during GI tract development in the chick embryo is the homeotic gene \textit{Bapx1} (also named Nkx3.2). \textit{Bapx1} expression has been reported for the vertebrate stomach (Nielsen et al., 2001; Akazawa et al., 2000; Verzi et al., 2009). In chick, \textit{Bapx1} is expressed early during development in the gizzard mesenchyme but not in the proventriculus (Nielsen et al., 2001). Ectopic expression of \textit{Bapx1} in the proventriculus results in a gizzard-like morphology and inhibits normal proventricular
expression of Bmp4, strongly suggesting a role for Bapx1 in the regionalization of the chick stomach through the control of Bmp4 expression (Nielsen et al., 2001; de Santa Barbara et al., 2005). In addition, it has been shown that when Bmp4 is misexpressed in the gizzard mesoderm, smooth muscle differentiation is impaired (Smith et al., 2000; de Santa Barbara et al., 2005). A regulated level of BMP signaling activity is then a prerequisite for normal development and differentiation of the gizzard mesenchyme (de Santa Barbara et al., 2005; Notar nicola et al., 2012). Although it has been shown that the ectopic expression of Bapx1 causes a marked muscular hypertrophy (Nielsen et al., 2001; de Santa Barbara et al., 2005), its expression profile during the differentiation of the mesenchymal layer into smooth muscle and submucosa remains to be examined.

The goal of this study was to analyze the endogenous expression pattern of Bapx1 during the process of smooth muscle differentiation, from E4 to E9, in the developing chick GI tract.

1. Results

1.1. Bapx1 expression in E4 chick GI tract

In order to investigate the expression pattern of Bapx1 during the development of the digestive tract, we first performed whole mount in situ hybridization analyses on 4-day-old dissected guts (E4 or stages 23–24) and observed that Bapx1 is expressed at high level in the stomach and the colon (Fig. 1A, C and E). Moreover, in situ hybridization analyses performed on paraffin sections revealed that Bapx1 expression is localized in the whole mesenchyme of the stomach and the colon (Fig. 1D and F). At these stages, the primitive gut presents no morphological differences along the anteroposterior axis. The whole digestive mesenchyme is undifferentiated as demonstrated by the absence of Sm22 expression, a specific differentiated smooth muscle cell marker (Fig. 1B). All together, these data demonstrate that Bapx1 is expressed at early stages of GI development. Its expression is localized in the mesenchyme of the stomach and the colon before their differentiation into smooth muscle.

1.2. Bapx1 expression in E7 chick GI tract

At E7 (Stage 31), the chick GI tract is regionalized into the stomach, the small intestine and the colon. Intestine and colon are separated by the bilateral caeca. The stomach is composed of two structures: the gizzard and the proventriculus, respectively the muscular and glandular stomach (Fig. 2A). At this stage, the mesenchyme of the GI tract is differentiated in smooth muscle as demonstrated by Sm22 staining uniformly localized into the GI musculature with the exception of the caeca and the two intermuscular tendons present in the stomach (Le Guen et al., 2009) (Fig. 2B). Whole-mount in situ hybridization analyses performed at this stage revealed a high level of Bapx1 expression in the gizzard (Fig. 2C and D). A strong expression of Bapx1 was also detected in the pylorus, a structure that allows the connection of the stomach to the duodenum (Fig. 2C). Immunostaining on paraffin sections indicated that at this stage, the mesenchyme of the stomach is differentiated along the radial axis into submucosa (negative for the specific alpha Smooth Muscle Actin marker (αSMA)) and smooth muscle (positive for αSMA) layers. The ent eric nervous system precursors (positive for HNK-1), organized in myenteric plexi, are localized in the outer part of the smooth muscle domain (Fig. 2E and H). Comparison of Bapx1 in situ staining to αSMA/HNK-1 immunostainings performed on adjacent longitudinal sections indicated that Bapx1 is highly expressed in smooth muscle cells in the gizzard (Fig. 2F; compare Fig. 2G and H). In addition, a faint expression of Bapx1 was also detected in the αSMA-negative submucosa layer. In the smooth muscle domain, Bapx1 expression was not detected in the plexi (Fig. 2G). At E7, the colon smooth muscle is well differentiated, as demonstrated by the expression of αSMA on paraffin sections (Fig. 2I). We found that Bapx1 is highly expressed in the colon (Fig. 2C, D and J). Interestingly, its expression is lower in the posterior part of the colon and even almost non detectable in the rectum (Fig. 2J). Analyses of Bapx1 staining on paraffin sections revealed that Bapx1 is localized in the submucosa layer with a higher level in cells of the submucosa layer bordering with the epithelium and the smooth muscle domain (compare Fig. 2I-K). These observations are confirmed by simultaneous in situ hybridization with Bapx1 coupled to immunofluorescence with αSMA analyses on the same colonic section (Fig. 2L).

1.3. Bapx1 expression in E9 chick GI tract

Whole-mount in situ hybridization analyses performed at E9 (Stage 35) revealed Bapx1 expression in the gizzard, the pylorus and the colon. No expression was detectable in the rectum (Fig. 3A and B). At this stage, we observed a new area of expression in the whole small intestine (see arrowhead Fig. 3A). As previously observed at earlier stages (see Fig. 2), the expression of Bapx1 in the gizzard is high and localized in the smooth muscle layers (compare Fig. 3C and D). A very faint staining was also detected in the submucosa layer (see magnified views in Fig. 3E and F). More posteriorly, we found that all smooth muscle layers (longitudinal, circular and oblique) are positive for Bapx1 (compare Fig. 3G and H). The expression of Bapx1 was also observed in the colon (Fig. 3I). At this stage, the colon harbors two αSMA positive smooth muscle cell domains that constitute, from outer to inner, the longitudinal and circular smooth muscle layers. The enteric neural crest precursors, positive for HNK-1, are organized into myenteric and submucosal plexi (shown respectively by white and red arrowheads in Fig. 3J). Comparison of Bapx1 in situ staining to αSMA/HNK-1 immunostainings performed on adjacent longitudinal sections revealed that Bapx1 is expressed both in the submucosa and the smooth muscle layers, with a stronger expression in cells located at the junction between these two domains (compare Fig. 3J and K). Interestingly, we observed that the width of Bapx1 expression domain in the smooth muscle decreases along the AP axis of the colon (Fig. 3I). Only a stripe of Bapx1 expression was found in a group of cells bordering the submucosa layer at the transition between colon and rectum (compare Fig. 3L and M) consistent with the absence of Bapx1 expression observed in the rectum (arrows in Fig. 3B). In the small intestine, the expression of Bapx1 is mainly localized in the submucosa layer and in the outer part of the undifferentiated intestinal mesenchyme (see respectively black and white arrows, Fig. 3N and O). These observations are confirmed by simultaneous in situ hybridization with Bapx1 coupled to immunofluorescence with αSMA analyses on the same section (Fig. 3P). Interestingly, no expression was detectable in the intestinal smooth muscle (compare Fig. 3N-P).

2. Discussion

It was previously shown that a low level of BMP signaling activity is a prerequisite for normal development and differentiation of the digestive mesenchyme (Roberts et al., 1998; Smith et al., 2000; de Santa Barbara et al., 2005; Notar nicola et al., 2012). A candidate gene for regulating BMP signaling during GI tract development in the chick embryo is the homeotic gene Bapx1 (or Nkx3.2) (Nielsen et al., 2001). Both gain-and loss-of Bapx1 functions have suggested a role for this gene in regulating the expression and patterning of
Bmp4 in the chick stomach (Nielsen et al., 2001). In this study, we report the endogenous expression pattern of Bapx1 during the chick GI tract development and digestive smooth muscle differentiation.

We found that Bapx1 is expressed in the mesenchyme of the gizzard during the early stages of GI development and that its expression is maintained in the mesenchyme during its differentiation into smooth muscle. Later on, Bapx1 expression is localized in all the smooth muscle layers (longitudinal, circular, and oblique) of the gizzard. The expression of Bapx1 in the smooth muscle of the stomach is maintained during development as we observed its expression at E18 (data not shown), when the digestive musculature is functional (Notarnicola et al., 2012).

In addition, we found an unexpected expression of Bapx1 in the pylorus, a conserved structure that connects the stomach to the duodenum. The development of the pyloric structure is controlled by the activity of the BMP signaling pathway (Smith and Tabin, 1999; Smith et al., 2000; Moniot et al., 2004). It has been reported that a sustained expression of Bapx1 in the pylorus induces a hypertrophic pyloric spincter, highly related to the Infantile Hypertrophic Syndrome observed in humans (Roberts et al., 1998; de Santa Barbara et al., 2002, 2005; Smith and Tabin, 1999; Moniot et al., 2004). Taken together, these results suggest that the endogenous expression of Bapx1 has to be tightly regulated during the development of this structure.

In this study, we also report an early expression of Bapx1 in the colonic mesenchyme. This observation is unexpected, as it has been shown that Bmp4 is strongly expressed in the colon at this early stage of GI tract development (Roberts et al., 1998; Smith et al., 2000; our unpublished results). Later during the differentiation of the colonic mesenchyme, Bapx1 is highly expressed in the submucosa layer, a domain composed of loose cells that express numerous extracellular matrix proteins such as laminin and fibronectin (Simon-Assmann et al., 1994). To our knowledge, Bapx1 is the first transcription factor described so far to be expressed in the submucosa layer of the colon. At later stage (E9), Bapx1 is expressed in the smooth muscle and the submucosa layer of the colon. Interestingly, we observed a gradient of Bapx1 expression along the AP axis in the colonic smooth muscle. Posteriorly, only a stripe of Bapx1 expression was found in the smooth muscle, in a group of cells bordering the submucosal layer. The expression of Bapx1 in the inner-most part of the muscle layer in the colon was also observed at E18 (data not shown).

All together, our study provides new perspectives concerning the roles of Bapx1 during the development and differentiation of digestive musculature.
3. Experimental procedures

3.1. Chick embryos

Timed fertilized white Leghorn eggs (Haas Farm, France) were incubated at 38°C in a humidified incubator (Coudelou, France) until used experimentally. Embryos were staged according to Hamburger and Hamilton (1951) by embryonic day (E) for gastrointestinal tract analysis. Dissected gastrointestinal tissues were fixed in 4% paraformaldehyde for 1 h at room temperature, washed in PBS, gradually dehydrated in Methanol in order to store the samples at 0°C before processing for whole-mount in situ hybridization as described below (Faure et al., 2002).

3.2. Whole-Mount in Situ hybridization

Whole-mount in situ hybridization analyses were carried out as described by Moniot et al. (2004) using antisens Bapx1 (Nielsen et al., 2001) and Sm22 riboprobes. Chick Sm22 cDNA was isolated, sequenced and used to prepare riboprobes for in situ hybridization using specific primer pair of chick Sm22 (Gene Bank ID: NM_205494.1; forward: 5'-TGA GCA GGG AGT TCC ACT -3', reverse: 5'-AGC CAA TGA TGT TCT TGC C-3') generated a 501 bp fragment cloned into the pGEMT-Easy plasmid (Invitrogen). Tissues were gradually rehydrated in PBS, washed in PBT (PBS, 0.1% Tween) and incubated in 6% hydrogen peroxide (Sigma, France). Samples were next permeabilized by treatment with proteinase K (10 l g/ml) for 10 min, washed with glycine in PBT and fixed in 4% paraformaldehyde/0.2% gluteraldehyde in PBT for 20 min. Tissues were then hybridized with antisens Bapx1 or Sm22 digoxigenin-labelled (Roche) riboprobes overnight at 70°C. After post-hybridization washes at 70°C, tissues were incubated in 10% sheep serum for 2.5 h at room temperature and finally mixed with preabsorbed anti-digoxigenin coupled with alkaline phosphatase antibody (Roche) overnight at 4°C. The complexes were detected with BM purple solution (Roche).

Fig. 2. Bapx1 expression in E7 chick GI tract. (A) Scheme illustrating the regionalized GI tract. (B) Whole mount in situ hybridization analysis of Sm22. Ventral view. Sm22 is expressed in the whole GI tract with the exception of the two intermuscular tendons (arrowheads) and the caecum. (C and D) Whole mount in situ hybridization analysis of Bapx1. Ventral (C) and dorsal (D) views. Bapx1 is highly expressed in the gizzard and the colon. (E) Immunofluorescence analysis performed on longitudinal paraffin sections using anti-aSMA and anti-HNK-1 antibodies showing respectively digestive smooth muscle cells (red) and enteric nervous cell precursors (green) organized in myenteric plexi. (F) In situ hybridization analysis of Bapx1 expression directly performed on adjacent longitudinal sections. In the stomach, Bapx1 is expressed in the smooth muscle cells in the gizzard and the pylorus. (G) Magnified view of the stomach shown in F (black arrow). Bapx1 is expressed in the smooth muscle domain. (H) Magnified view of the stomach shown in E (white arrow). White arrowhead indicates myenteric plexi. (I) Immunofluorescence analyses performed on longitudinal paraffin sections using anti-aSMA antibody. (J) Magnified view of the colon shown in C and D. (K) In situ hybridization analysis of Bapx1 expression directly performed on sections. In the colon, Bapx1 is mainly expressed in the submucosa layer. Note the high level of Bapx1 expression in a group of cell bordering both the epithelium (black arrowhead) and the smooth muscle domain (red arrowhead). (L) Longitudinal paraffin section processed for both in situ hybridization (using Bapx1 riboprobe) and immunofluorescence (using anti-aSMA antibody) analyses. Bapx1 is expressed in the submucosa layer adjacent to the smooth muscle domain that is positive for aSMA. Abbreviations: cae, caecum; co, colon; e, epithelium; gz, gizzard; m, smooth muscle layer; py, pylorus; rect, rectum; sm, submucosa; st, stomach.

3.3. Immunohistochemistry and in situ hybridization on paraffin sections

For sectioning, tissues were first gradually dehydrated in Ethanol, incubated in HistoClear solution (VWR, France) and finally embedded in Paraffin. Samples were sectioned at 10-um using a microtome and collected on Polysine coated-slides (Thermo Fisher). In situ hybridization experiments on paraffin sections were carried out as previously described (Moniot et al., 2004) using antisens Bapx1 riboprobes (Nielsen et al., 2001). Immunofluorescence
studies were performed on paraffin sections using polyclonal antibodies against αSMA (Sigma–Aldrich) and HNK-1 (NeoMarker). Nuclei were labelled with Hoescht (Invitrogen).

3.4. Photography

Images of whole-mount GI tract staining were acquired using a Nikon-AZ100. Images of sections were acquired using a Nikon AZ100 microscope.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version,

References