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Cdx2 initiates histodifferentiation of the midgut endoderm

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Abstract **Null mutation or haploinsufficiency of *Cdx2* results in the development of heterotopic lesions with a gastric phenotype in the midgut endoderm. Conversely transgenic expression of *Cdx2* in the stomach causes the endoderm to differentiate into intestinal-type mucosa. We demonstrate that the mesoderm adjacent to intestinal heterotopic areas expresses stomach specific *Barx1* while the surrounding mesoderm is *Barx1* negative. We conclude that the initiation of gut histodifferentiation lies in the endodermal expression of *Cdx2* and that endodermal/mesodermal cross-talk involving *Barx1* with appropriate feedback loops results in the development of the postnatal gut phenotype.**

Keywords: Gut histodifferentiation; *Cdx2*; *Barx1*; *Sox2*

1. Introduction

Cdx2 is expressed throughout the endoderm of the intestine distal to the developing stomach from the appearance of the head and tail folds [1]. It persists throughout life and is found at highest levels just distal to the apex of the midgut loop diminishing both cranially and caudally but never appearing in the stomach.

In vivo knock out [2] and overexpression studies [3] indicate that the gene is involved in morphogenesis of the intestinal epithelium. Chimaeric mice bearing $Cdx2^{-/-}$ cells in wild-type hosts, develop intestinal patches of organotypically normal stomach epithelium [4] and in $Cdx2^{+/-}$ animals areas of stomach mucosa develop in the paracaecal region [5], this being the area in which the gene is normally required at its highest level for normal development and therefore that in which haploinsufficiency is most likely to manifest. Clonal analysis in male wild-type animals bearing female $Cdx2^{-/-}$ lesions shows that the gastric heteroses are of chimaeric (female) origin while the underlying stroma is of host (male) origin [4]. Thus, *Cdx2* is required for the histogenesis of intestinal epithelium and in its absence or haploinsufficiency development along a 'default' pathway results in the formation of a region of stomach mucosa.

This model allows study of the epithelial/mesenchymal interaction necessary for normal intestinal morphogenesis. Mucosal intestinal recombination experiments have yielded conflicting results. For example, recombination of E14 distal endoderm with proximal mesoderm in mouse foetal tissue results in a small intestinal phenotype but conversely proximal endoderm recombined with distal mesoderm continues to differentiate as small intestine [6,7]. In the present experiments, we ask whether the mesoderm underlying areas of paracaecal gastric heteroses expresses genes normally absent from the mesenchyme of the small intestine but present in the stomach mesoderm. The demonstration of such gene expression would prove the existence of mesenchymal responsiveness to overlying gastric endodermal signals and indicate that the primary signal for gut differentiation is of endodermal origin.

We have chosen the gene *Barx1* as a marker of gastric mesoderm since it is involved in defining the stomach phenotype. Kim et al. [8] have shown that it is expressed in the mesoderm of the developing stomach and not in that of the intestine. Furthermore, null mutation of *Barx1* results in underdevelopment and malrotation of the stomach together with a disorganisation of the gastric mucosa and an anterior homeotic shift of the duodenum [9].

2. Materials and methods

2.1. Animals

$Cdx2^{+/-}$ embryos were obtained from matings of $Cdx2^{+/-}$ [10] males with C57BL6 females and timed from the morning of the day when a vaginal plug was found. $Cdx2^{-/-}$ chimaeric embryos were obtained as described previously [4].

2.2. RT-PCR

Intestines were dissected and RNA extracted using an RNeasy kit (Qiagen). RNA was reverse transcribed using oligo-(dT) and Superscript™ III reverse transcriptase (Invitrogen). Reddy mix (AB gene) was used for the amplification of *Barx1* and *Gapdh* and PuRe Taq Ready-To-Go™ PCR Beads (GE Healthcare) for *Sox2* amplification. Primers were; *Barx1*: 5′-GCCTGAGCCAGTTACAGGTG-3′ and 5′-TGTCTCTTCGGTCGCCTCTG-3′, *Sox2*: 5′-TAGCACTTGTTGCCAGAACG-3′ and 5′-CCTTTTCTCTTCTCGCCGAA-3′ [11], *Gapdh*: 5′-AGGTCGGTGTGAACGGATTTG-3′ and 5′-ACTGGAGTTGATGTACCAGATGT-3′. Cycling parameters were: 35 cycles of 95 °C (30 s), 61.1 °C (*Barx1* and *Sox2*) or 55 °C (*Gapdh*; 30 s) and 72 °C (1 min).

2.3. In situ hybridisation and immunohistochemistry

Whole mount in situ hybridisation with digoxigenin probes was as described [12]. *Sox2* probes were generated from an I.M.A.G.E. clone (MRC Geneservice: I.D. 6413283) and the *Barx1* probes from a plasmid donated by Prof. P. Sharpe. Sections following DIG labelling were counterstained with Nuclear Fast Red or Haematoxylin and Eosin (H&E). For radiolabelling, embryonic intestines were fixed in 4% paraformaldehyde or methacarn, embedded in 2% agarose and subsequently in paraffin, and serial sectioned at 8 μm. Adjacent sections were hybridised with *Sox2* or *Barx1* ^{35}S-UTP-radiolabelled probes. Slides were dipped in Ilford K5 Nuclear Emulsion (Agar Scientific)

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and left in the dark for 10 days before development in Kodak D-19 developer. Slides were counterstained with 1:100 Giemsa solution for 1 min. Adjacent sections were processed for immunohistochemistry with a Cdx2 antibody as described [1,13].

3. Results

3.1. Nascent areas of heterotopic endoderm can be localised using Sox2 as a marker

Sox2 is expressed in the stomach of the embryo [14]. Within the stomach, *Sox2* expression initially extends up to, but not including, the duodenum. Heterotopic lesions of stomach endoderm in the paracaecal region of the E12–E16 embryos cannot be detected histologically. *Sox2* was therefore used to determine their position. Whole mount in situ hybridisation was chosen for this purpose because it allowed the entire gut to be investigated at once. Hybridisation of the embryonic stomach indicated the specificity of the Sox2 probe (see Fig. S1 in the supplementary material). In $Cdx2^{+/-}$ gut at E12.5, E14.5 and E16.5 we detected on average 8 circumscribed regions of *Sox2* expression (Fig. 1A–D). We also detected discrete areas of *Sox2* expression in the midgut of an E12.5 $Cdx2^{-/-}$//WT chimaera (Fig. 1E).

3.2. Barx1 expression can be detected in the midgut of embryos expressing ectopic Sox2

Since *Sox2* expressing areas of heterotopia were detected in $Cdx2^{+/-}$ and $Cdx2^{-/-}$//*WT* embryos, the nature of the gut mesoderm was investigated. Using expression of the *Barx1* gene as a marker for stomach mesoderm, we first performed semi-quantitative RT-PCR. RNA was prepared from the paracaecal region of $Cdx2^{+/-}$ embryos at E14.5 and E16.5 and was subjected to RT-PCR using primers for *Sox2*, *Barx1* and *Gapdh* as a control. *Barx1* and *Sox2* transcripts were detected in E14.5 $Cdx2^{+/-}$ midgut (Fig. 2) and similar results were obtained in E16.5 $Cdx2^{+/-}$ midgut (data not shown). At these stages the heterotopic lesions are very small (Fig. 1) so negative PCR results were obtained in some embryos.

3.3. Barx1 expression mirrors Sox2 expression

To further investigate *Barx1* expression, whole mount in situ hybridisation with a *Barx1* DIG-labelled RNA probe was

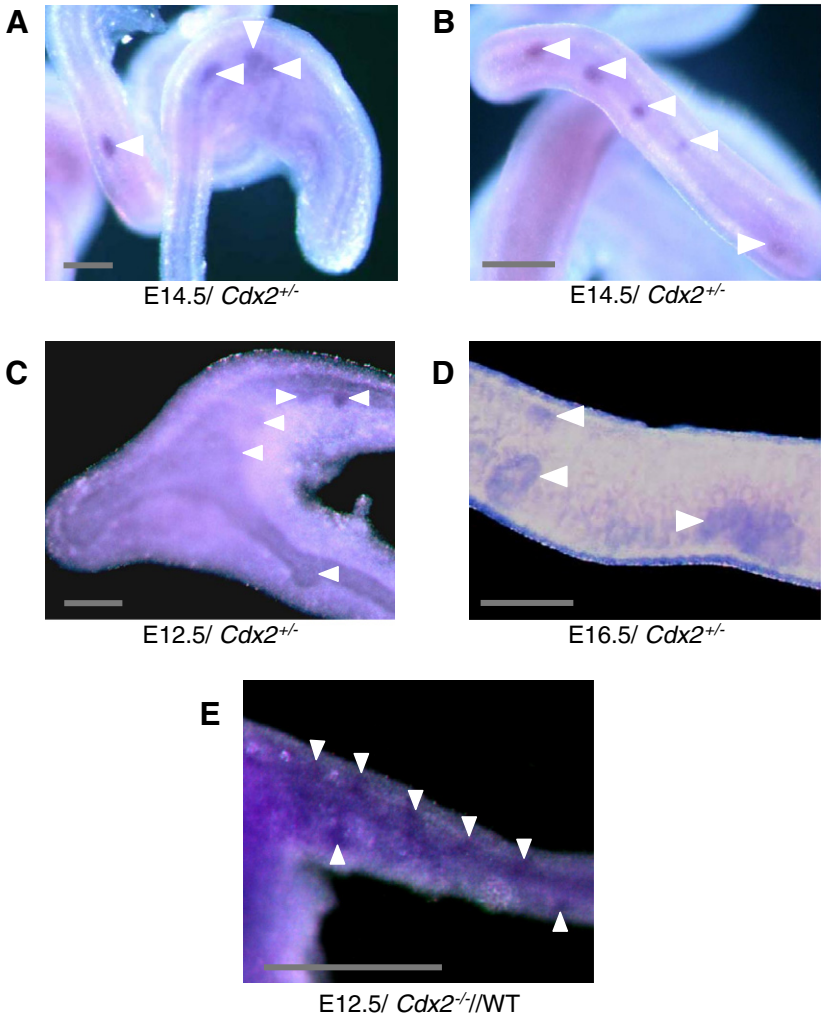

Fig. 1. Detection of areas of heterotopia using *Sox2*. Whole mount in situ hybridisation using a DIG-labelled *Sox2* antisense RNA probe to detect nascent areas of heterotopia. (A) E14.5 $Cdx2^{+/-}$ embryonic caecum; (B) E14.5 $Cdx2^{+/-}$ embryonic small intestine; (C) E12.5 $Cdx2^{+/-}$ embryonic caecum; (D) E16.5 $Cdx2^{+/-}$ embryonic small intestine; (E) E12.5 $Cdx2^{-/-}$//WT embryonic proximal colon. The arrows indicate regions of *Sox2* expression; these regions are deduced to be regions of stomach endoderm developing in the intestine. Scale bars = 500 μm.