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A study of regional gut endoderm potency by analysis of *Cdx2* null mutant chimaeric mice

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Abstract

Inactivation of *Cdx2* by homologous recombination results in the development of forestomach epithelium at ectopic sites in pericaecal areas of the midgut of heterozygote mice. Local factors subsequently result in the secondary induction of tissues exhibiting an orderly sequence of tissue types between the ectopic forestomach tissue and the surrounding colon. Clonal analysis of this secondarily generated tissue using Y chromosome painting in chimaeric mice indicates that once differentiated to express *Cdx2*, host colonic epithelium can only form small intestinal-type epithelium, while *Cdx2* mutant cells give rise to a succession of gastric-type tissue but never to a small intestine morphology. Our results indicate a difference in potency between forestomach and midgut precursor endodermal cells.

Keywords: *Cdx2*; Homeobox; Intercalation; Gut development

Introduction

The mouse homeobox gene *Cdx2* is one of the homologues of the *Drosophila* gene *caudal*. First isolated by Mlodzik and co-workers (Mlodzik et al., 1985) *caudal* belongs to the hexapeptide class of homeobox genes (Burglin, 1994) but is not a member of the HOM cluster. In *Drosophila*, the gene is initially necessary for normal specification of the antero-posterior axis of the organism and subsequently its action in combination with locally produced factors specifies the development of the components of the Analia into the anal plate and posterior gut (Moreno and Morata, 1999). In late embryonic, postnatal, and adult mice Cdx2 expression is confined to the epithelial cells lining the intestine. Highest levels are found in the proximal colon, tapering off both proximally and distally (James and Kezenwadel, 1991) but the protein is immunocytochemically detectable in all regions of the postgastric intestinal epithelium throughout life. The action of *Cdx2* shows similarities to those of *caudal* insofar as animals in which one copy of the gene has been inactivated by homologous recombination exhibit an anterior homeotic shift involving the axial skeleton while regional changes in the midgut result in the appearance of forestomach epithelium in the mucosa of the terminal ileum, caecum, and proximal colon (Chawengsaksophak et al., 1997). Furthermore, local factors lead to sequential inductive interactions between the forestomach epithelium and the surrounding midgut mucosa (Beck et al., 1999). As a result, and following the establishment of forestomach epithelium, a succession of histological tissue types develop between the ectopic forestomach and the surrounding colon. Thus, an ordered series characteristic of cardia, stomach body, pyloric antrum, and small intestine arises by secondary induction between the forestomach epithelium and surrounding colonic epithelium as development proceeds.

The homozygous *Cdx2*−/− phenotype is embryolethal, but the creation of chimaeras of homozygous null mutant cells in wild-type recipients makes it possible to investigate the clonal nature of the tissue that is secondarily induced

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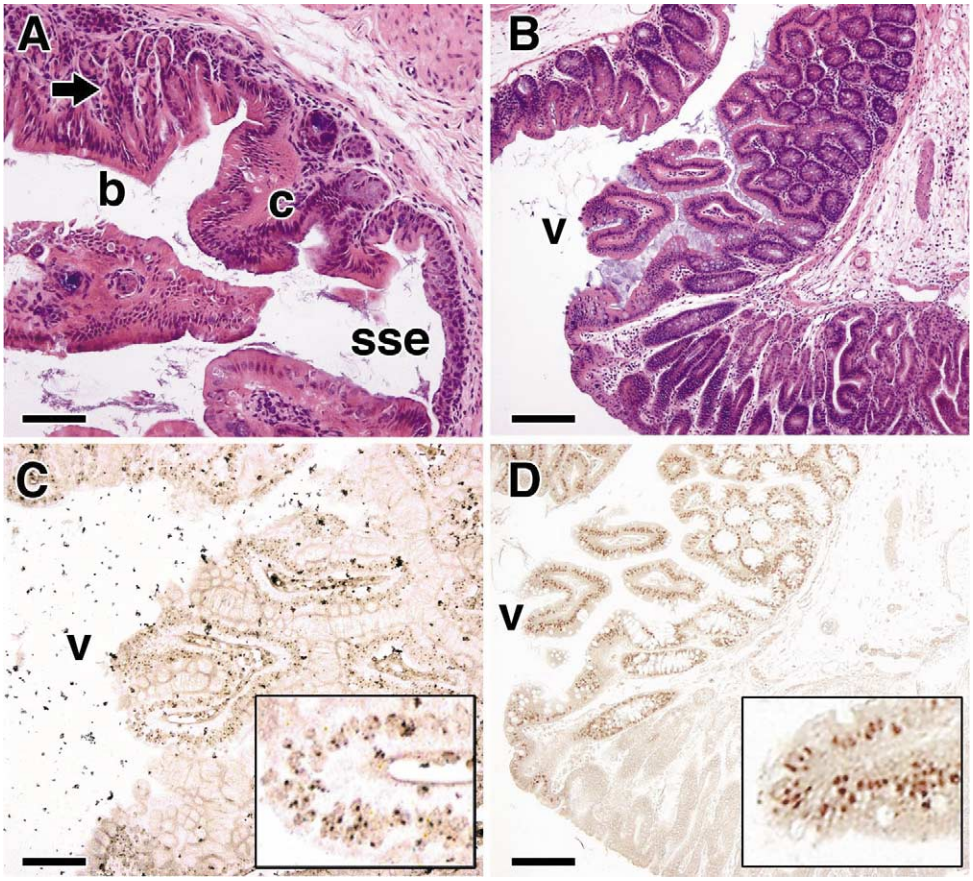

Fig. 1. Sections cut at 5 μm through caecal polyps from a *Cdx2*−/− wild-type chimaera. (A) Stained with H&E, showing stratified squamous epithelium (sse) adjacent to mucus secreting cardia (c), which in turn gives way to mucosa characteristic of stomach body (b) containing clearly defined oxyntic cells (at arrow) (bar = 75 μm). (B) Villous structures (v) found in the peripheral regions of the polyps are seen to be covered by enterocytes with a well-marked brush border and by goblet cells (bar = 150 μm). (C) A higher power view of the villous structures in the adjacent section stained to show Y chromosomes reveals that the enterocytes clothing the villi in this male chimaera are clearly Y-positive (bar = 75 μm). Inset, ×3. (D) A further adjacent section stained for Cdx2 reveals that the polyp tissue is Cdx2-negative, with the exception of the peripheral villous-type region which, similar to the adjacent normal large intestine, is Cdx2-positive (bar = 150 μm). Inset, ×3.

between the "default" forestomach epithelium generated by *Cdx2*-deficient cells and the surrounding host (wild-type) colonic epithelium. It thus becomes possible to determine the developmental potential of *Cdx2*-expressing and *Cdx2*-null cells in the context of gut differentiation. We ask whether the various tissue types secondarily generated between forestomach and colon arise from the chimaeric *Cdx2*−/− cells, the host epithelium, or both. Using Y chromosome painting to determine cell origin together with Cdx2, sucrase isomaltase, and intestinal fatty acid binding protein (FABPI) staining to indicate tissue types, we demonstrate that host colonic epithelium can only form small intestinal-type epithelium, while *Cdx2*-negative chimaeric cells give rise to all the remaining tissue types but never to a small intestinal morphology. Our results suggest a fundamental difference in the potency of forestomach and midgut precursor endodermal cells; each is able to generate the cell types of its own gut region but is unable to differentiate into cells of the adjacent region.

Materials and methods

Derivation of ES cell lines from Cdx2 heterozygote intercross embryos

Cdx2−/− ES cell lines were derived from blastocysts of mixed background (129SV × C57BL/6J) *Cdx2*+/− heterozygote intercrosses. The protocol used has been described (Abbondanzo et al., 1993). Briefly, 4-day blastocysts were flushed from the uterus, hatched by exposure to acid tyrode (Hogan et al., 1994), washed several times in M2 medium, and seeded onto a gelatinized four-well plate containing mouse primary embryonic fibroblasts. Following undisturbed culture for 4 days, each ICM was picked using a flame-drawn mouth pipette, washed twice in sterile PBS, placed in a single trypsin microdrop (25% trypsin, 1 mM EDTA (CSL), 1% chicken serum (TRACE)), covered with mineral oil, and incubated at 37°C for 5 min. The loosened ICM was then broken up to a single-cell suspension by

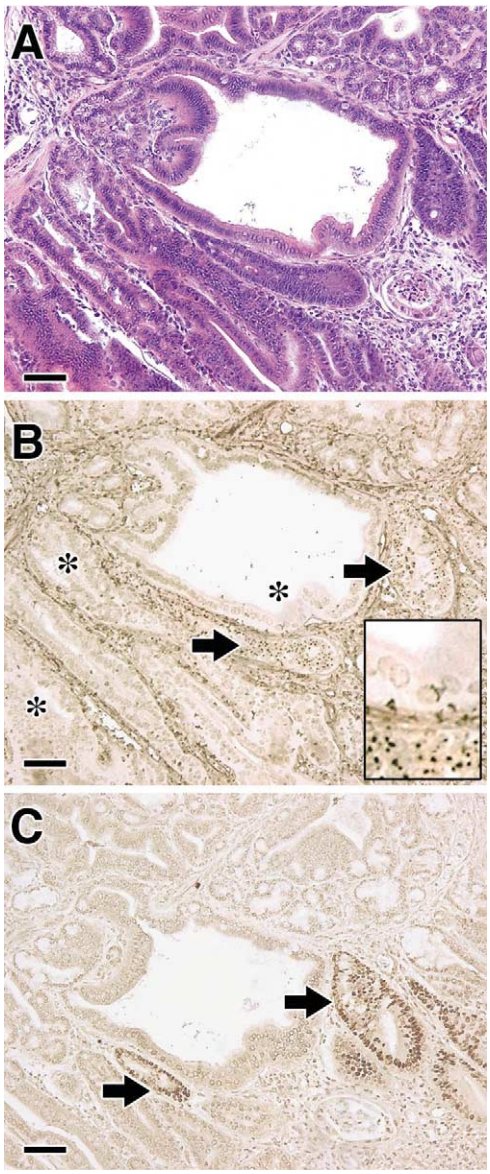

Fig. 2. Sections cut at 5 μm through the interior of caecal polyp from a male *Cdx2*−/− wild-type chimaera containing "trapped" areas of normal colon. (A) Stained with H&E. (B) An adjacent section stained to show Y chromosomes, revealing that trapped areas of normal colon from male host are Y-positive (at arrows), whereas chimaeric (female) endodermal cells making up the substance of the polyp are Y-negative (*) (bar = 75 μm). Inset, ×3. Stromal cells, which are of host origin, are Y-positive throughout. (C) An adjacent section stained for Cdx2 reveals that the endodermal cells of the polyp are Cdx2-negative, whereas trapped regions of normal colon are Cdx2-positive (arrow) (bar = 75 μm).

pipetting up and down with a flame-drawn mouth pipette and transferred to a single well of a 16-mm tissue culture dish containing mouse embryonic fibroblasts. Between 10 and 30% of the preparations developed an ES cell morphology within 3 to 4 days after disaggregation of the ICM. These were expanded and frozen in liquid nitrogen as described (Abbondanzo et al., 1993). Karyotyping showed that the *Cdx2*−/− cell line used in the present experiments was female with a normal chromosome complement.

Generation of antibodies to Cdx2

The nucleotide sequence 1–327 that codes for the unique first 109 amino acids of the Cdx2 peptide was cloned into the expression vector pET15b (Novagen) and sequenced. Expressed protein was purified and antibodies were raised in rabbits. Crude serum was tested on Western blots of nuclear extracts from CaCo2 cells and shown to detect a protein of the size corresponding to Cdx2. Strong nuclear staining was observed in the midgut epithelium of wild-type mice and in "host" (i.e., Y-positive) midgut epithelium of male chimaeras while Cdx−/− (Y-negative) chimaeric cells did not stain.

Histology, immunohistochemistry, and in situ hybridisation

For routine histology, tissues were immersion-fixed in 4% (wt/vol) paraformaldehyde, embedded in paraffin by standard methods, cut into 5-μm sections, and stained with haematoxylin and eosin or by Mowry's (Mowry, 1956) technique to identify intestinal mucins.

Immunohistochemical detection of Cdx2 was carried out according to the procedure described in Beck et al. (1995).

A monoclonal antibody (IGM) raised against part of the sequence of human trefoil factor family 2 (TFF2) (Elia et al., 1994) was used to detect expression of this major antral peptide (Hanby et al., 1993) using an indirect Avidin biotin peroxidase complex and 3,3′-diaminobenzidine to generate a brown signal.

A Y chromosome paint, labelled with FITC (Cambio, Cambridge, UK), was used to locate the Y chromosome and was detected using an anti-FITC peroxidase conjugate (Roche) as described in Poulsom et al. (2001).

Sections of formalin-fixed paraffin-embedded tissues were processed as described (Poulsom et al., 1998), using riboprobes labelled with ^{35}S-UTP. A near full-length antisense riboprobe for mouse sucrase isomaltase was generated using a T7 RNA polymerase-linearised plasmid. Autoradiographic exposure was for approximately 10 days, before development in Kodak D19 and counterstaining in Giemsa's stain. Sections were examined under conventional and reflected light–dark field conditions.

Results

Chimaeric animals have polypoid lesions in the midgut

Chimaeras were made by injection of *Cdx2*−/− ES cells into MF1 white (outbred) recipient blastocysts. The *Cdx2*−/− cells were obtained by selection of homozygous mutant embryos from heterozygote intercrosses and injected. Initially, a series of 23 recipient mice received blastocysts each injected with 16 mutant ES cells. Only 11 of these established pregnancies, which survived to full term, implying that a large complement of *Cdx2*−/− cells in

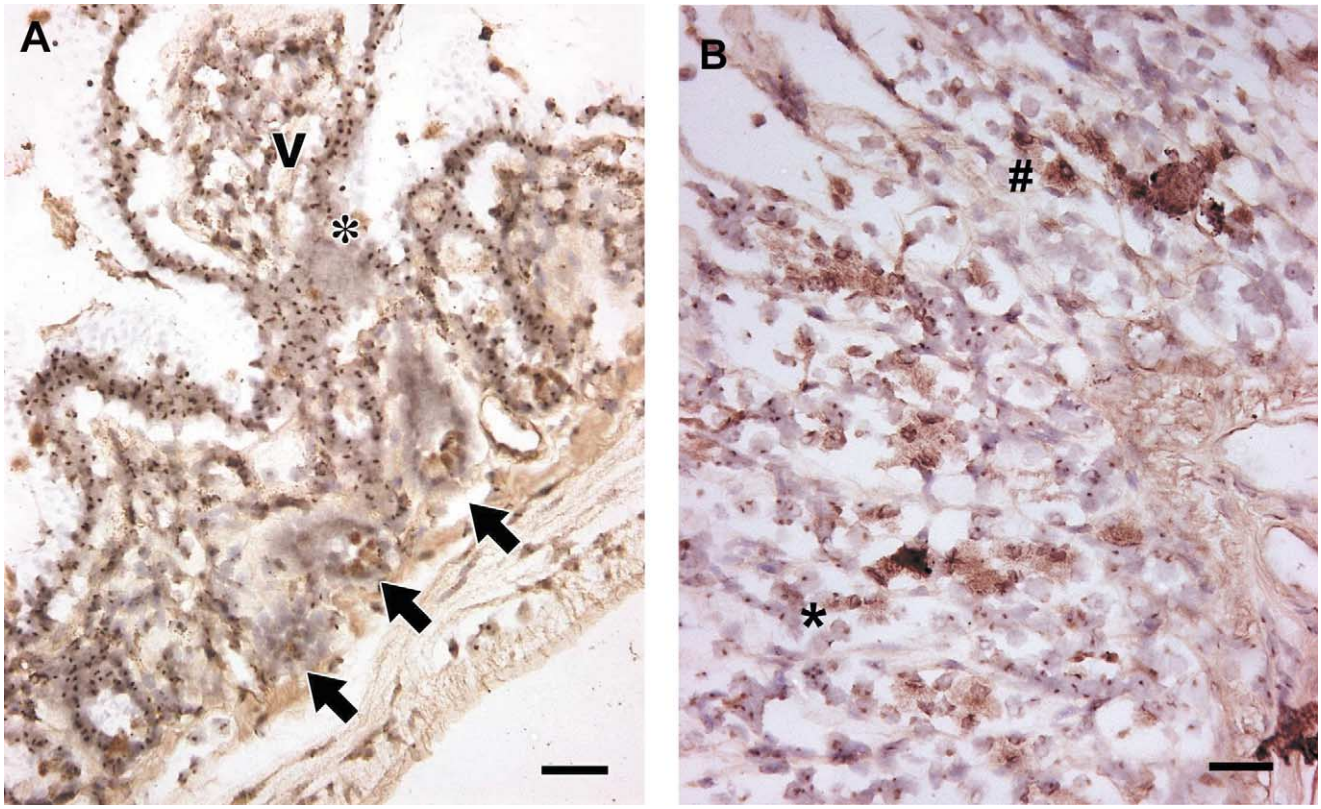

Fig. 3. (A) A section through a macroscopically normal portion of the distal jejunum from a male *Cdx2*−/− wild-type chimaera stained to show Y chromosomes. Three apparently normal Y chromosome-negative crypts (arrows) are seen flanked by Y-positive crypts; part of the villus surface epithelium (v) is appropriately negative (*) (bar = 37.5 μm). (B) Section through a macroscopically normal portion of stomach mucosa from a male *Cdx2*−/− wild-type chimaera stained to show Y chromosomes. Tissue on the upper part of the illustration (#) is clearly Y-negative and therefore of chimaeric origin, while that in the lower part of the section (*) is Y-positive and of host origin (bar = 37.5 μm).

chimaeras, was incompatible with survival. Subsequent experiments therefore were performed with injection of between six and eight null mutant cells into each blastocyst. Of 24 blastocyst transfers, 21 established successful pregnancies that went to full term. In total, 31 chimaeric animals (as assessed by coat colour) aged between 19 days and 18 months were examined in detail.

With seven exceptions, the intestines of all the chimaeric animals were found to have polypoid lesions in the midgut. The principal location was in the caecum, with occasional polyps in the distal ileum and/or the proximal colon. Between one and three such sites were present in each animal and sometimes they overlapped. Microscopic examination revealed that the lesions corresponded to those whose histology and ontogeny was described by Beck et al. (1999). As before, the lesions developed spontaneously without evidence of trauma or infection. Thus, areas of keratinising stratified squamous epithelium were present in the substance of the polyps, next to which there was usually a region of neutral-mucous-secreting epithelium characteristic of gastric cardia, followed in sequence by body of stomach (with characteristic oxyntic cells, gastric enterochromaffin cells, etc.) and pyloric antrum (Fig. 1A). At the periphery there was usually a region exhibiting a villus-like morphology bearing enterocytes with well-marked brush borders together with goblet cells, but no Paneth cells were seen in any of the lesions (Fig. 1B). The various tissue types were organotypically normal and there was no evidence of dysplasia.

Apart from the midgut lesions described above, there were no other heterotopias. Blastocysts injected with wild-type (*Cdx2*+/+) cells do not produce midgut lesions.

Clonal analysis of the polyps shows that the bulk of the lesion is made up of mutant cells while peripheral structures with a villous morphology are of host origin

The mutant ES cells used in this experiment were female as shown by karyotyping. Staining of male chimaeras with Y chromosome paint indicates that in all the polypoid lesions the areas with morphology indicative of oesophagus/forestomach, cardia, corpus, and pyloric antrum were of mutant origin, i.e., Y chromosome negative (Fig. 2B), but that the villous-like peripheral regions together with the areas displaying normal colonic morphology were of host origin, i.e., Y chromosome positive (Fig. 1C).

Within the polyps, the stromal cells underlying the epithelium were predominantly of host origin (male). Occa-

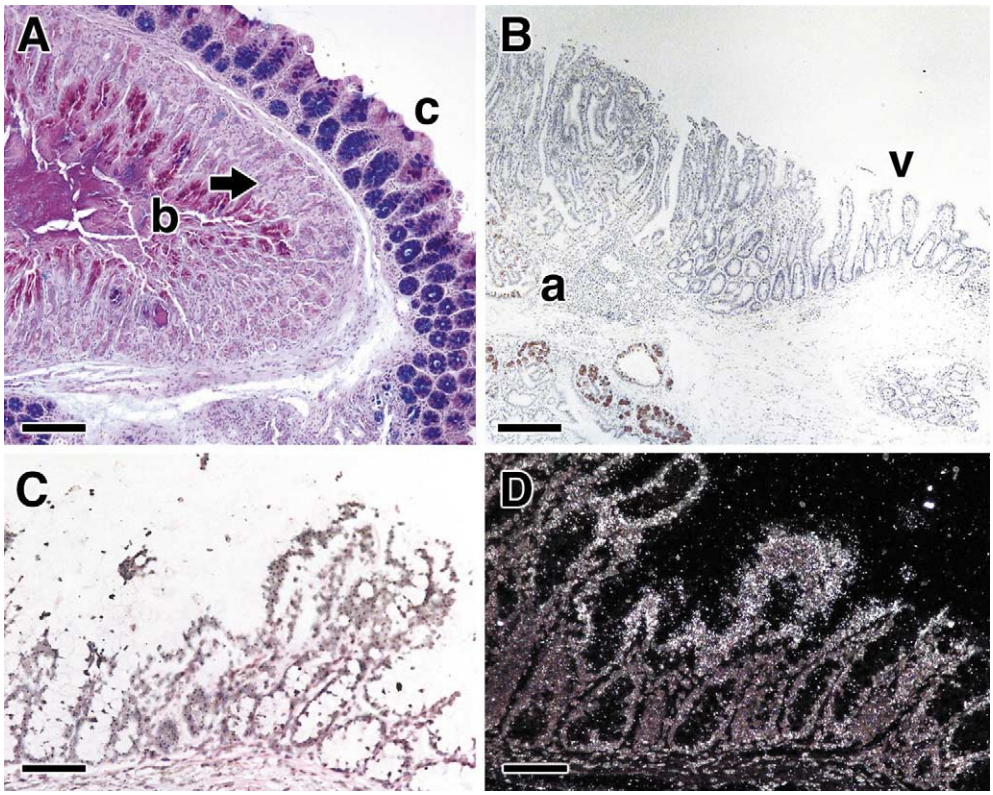

Fig. 4. Sections through a caecal polyp taken from a *Cdx2*−/− wild-type chimaera. (A) Using the Mowry technique, the normal colon (c) stains with Alcian blue, indicating the presence of acid mucopolysaccharides, whereas the polyp tissue which has the histological appearance of stomach body (b) is PAS-positive, denoting the presence of neutral mucopolysaccharides (bar = 150 μm). (B) Peripheral region of a caecal polyp stained to detect TFF2; gastric antrum-like glands (a) are seen to express this peptide, whereas the villous structures (v) do not (bar = 150 μm). (C) A nearby section stained to show Y chromosomes, revealing that the crypts and villi are Y-positive (bar = 50 μm). (D) A nearby section hybridised to an antisense riboprobe to detect sucrase isomaltase mRNA; white reflective silver grains reveal that the villous-like epithelium expresses this mRNA appropriately (bar = 50 μm).

sionally, areas of normal large intestine were "trapped" within the lesions. These secreted acid mucopolysaccharide, stained positively for Cdx2 (Fig. 2A and C), and had the morphology of colonic crypts. Without exception they were Y chromosome positive (Fig. 2A and B).

Examination of diverse organs in the chimaeric animals showed that the mutant cells were incorporated throughout the animal and formed normal structures, distinguishable from the host cells only by virtue of their Y chromosome staining patterns (Fig. 3A and B). We did not examine the axial skeleton, which was the only structure other than the intestine to be affected in *Cdx2*+/− heterozygotes (Chawengsaksophak et al., 1997).

Histochemistry and immunohistochemistry of polypoid lesions

After staining by the Mowry (1956) technique, the mucous-secreting cells present in the lesions displaying tissue characteristic of stomach morphology showed PAS-staining (neutral) mucopolysaccharides, whereas the proximate colonic mucous-secreting cells were Alcian blue staining, denoting the presence of acid mucopolysaccharide (Fig. 4A). Regions of the polyps that exhibited the histology characteristic of pyloric antrum stained positively for TFF2 (Fig. 4B).

Sections stained for Cdx2 showed that the "gastric" areas of the polyps were Cdx2-negative (Figs. 1D and 2C), a finding that is consistent with, but does not prove, that they were derived from mutant ES cells. Trapped areas of normal colonic tissue within the polyps were Cdx2-positive (Fig. 2C), as were the peripherally located villous-like structures, proving that they are of host origin (Fig. 1D).

Immunocytochemistry with an antibody to FABPI results in diffuse cytoplasmic staining of enterocytes clothing the villi of the normal small intestine, whereas cells in the colonic region generally do not stain. However, occasional FABPI-positive areas were detected in normal colonic tissue. The villous-like structures at the periphery of the caecal and colonic polyps were found to be FABPI-positive in contrast to the neighbouring negative cells of normal-looking large intestine. The more centrally located regions of gastric-type tissue were uniformly FABPI-negative (Fig. 5). In situ hybridisation for sucrase isomaltase mRNA, which codes for an enzyme restricted to small intestinal mucosa, also showed a positive reaction in the villous profiles at the

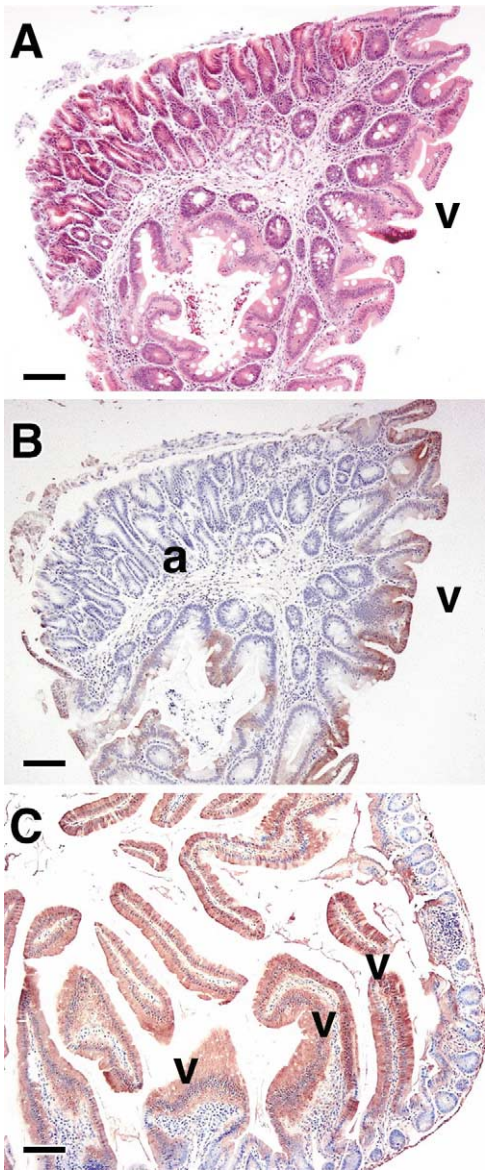

Fig. 5. Sections through a caecal polyp from a *Cdx2*−/− wild-type chimaera including the peripheral villous-type region. (A) Stained with H&E, revealing villous structures (V) (bar = 150 μm). (B) An adjacent section stained for FABPI showing a positive reaction in the enterocytes clothing the villi (v), and no reaction in the remainder of the polyp which has the histology characteristic of pyloric antrum (a) (bar = 150 μm). (C) Normal mouse small intestine stained for FABPI showing a positive reaction on enterocytes clothing the villi (v) (bar = 150 μm).

edges of the polyps, while both the gastric-type regions of the polyps as well as the surrounding normal colon were negative (Fig. 4C and D).

Discussion

It is established that *Cdx2* haploinsufficiency results in the abnormal differentiation of midgut endoderm (Chawengsaksophak et al., 1997). The initial effect, apparent in neonates, is seen as patches of forestomach epithelium present in the terminal ileum, caecum, or proximal colon (Beck et al., 1999). Thus, in Cdx2 deficiency, cells which would normally differentiate into caecum/proximal colon follow a default pathway and form the stratified squamous epithelium characteristic of forestomach. While examples of gut transdifferentiation such as Barrett's oesophagus (Barrett, 1957) and the development of gastric mucosa in Meckel's diverticulum (Sonderland, 1959) are well documented, our observations are unique insofar as an orderly progression of various tissue types develops secondarily between the default forestomach epithelium present at birth and the surrounding host colon.

In studying the inductive interactions which give rise to the intermediate cell types, we have performed a clonal analysis of the intercalated tissues to determine whether they originate from forestomach or colon-type epithelium or from both. The answer allows us to determine the prospective potency of each of these populations and may be of practical importance in the context of studying gut regeneration following injury.

In all cases, the substance of the polyps containing forestomach, cardia, stomach body, and antrum-like tissue was of chimaeric cell origin. Small trapped areas of colonic epithelium within the substance of some polyps were of host origin. They retained the histological appearance of colonic tissue and secreted acid mucopolysaccharides as indicated by Mowry staining. At the periphery of many such polyps areas of villous-like mucosa were seen. These had characteristics of small intestinal epithelium including a positive reaction for sucrase isomaltase mRNA on in situ hybridisation, and immunohistochemically demonstrable Cdx2. Y chromosome painting confirmed their host origin. Significantly, we did not see any Paneth cells in these regions and in this respect the polyps differed from those seen in *Cdx2*+/− heterozygotes. While this may be the result of sampling discrepancy, it is possible that the small and large intestinal phenotypes are not as clearly defined in developmental terms as their adult histology would suggest. The secondary induction referred to above might be sufficient to produce intestinal villi but other or additional influences might be required for the differentiation of Paneth cells.

Thus we find that the *Cdx2*-deficient cells which have formed areas of ectopic forestomach in the proximal large intestine are capable of transdifferentiating into all the organotypically separate regions of the stomach but are never able to give rise to tissue with a small intestinal-type phenotype. This is a feature of the positional "readout" locally operative in the intercalated tissue since *Cdx2*−/− cells are able to form normal small intestinal crypts when they find themselves in the duodenum, jejunum, or ileum (Fig. 3A). Conversely, host colon-type epithelium can transdifferentiate to form small intestine-type epithelium but is incapable of forming stomach tissue.

The orderly sequence of tissue types within the colonic lesions is probably the result of the establishment of a series of secondary inductive processes whereby one group of cells changes the behaviour of an adjacent set of cells during development (Holzer, 1968), provided the latter are competent to respond to the inductive influence (Waddington, 1940). It is emphatically not the result of varying levels of Cdx2 expression since the chimaeric cells are homozygous *Cdx2* mutants.

The identity of the agents responsible for the intercalation process is clearly of interest. Proximate interactions are usually mediated by paracrine factors and the major paracrine agents involved in development appear to fall into four groups (Gilbert, 1997). They are the Hedgehog family, the Wingless (*Wnt*) family, the *TGF-β* superfamily, and the fibroblast growth factor (*FGF*) family of growth factors. Members of each of these groups or a combination of them have been shown to be involved either in the regulation of *cad* homologues in vertebrates or in gut development.

Thus, Roberts et al. (1995, 1998) have shown that Sonic hedgehog (*Shh*) is expressed in the definitive endoderm at the earliest stage of chick gut formation; they also demonstrated that *Shh* induces *Bmp4* (a member of the *TGF-β* superfamily) in midgut and hindgut mesoderm but not in the stomach region of the foregut. *Shh* also induced *Hox-d13* expression in the hindgut and might thus be implicated in anteroposterior gut patterning by this route.

Lickert et al. (2000) demonstrated that undifferentiated intestinal endoderm responded to *Wnt1* signals by upregulating expression of *Cdx1* although they were unable to elicit this response with *Cdx2*. More recently Lickert et al. (2001) described specific, temporally limited, *Wnt* gene expression in mouse gut development—a result which suggests that *Cdx2* may be regulated by a different combination of *Wnt* signals to that operative for *Cdx1*.

Pownall et al. (1996) and Isaacs et al. (1998) have shown that *Xcad3* is an intermediate early target of the FGF signalling pathway in *Xenopus* development, that normal expression of posterior *Hox* genes is dependent on FGF signalling, and that this regulation is probably mediated by the activation of *Xcad3*.

Intercalation does not occur when "incompatible" gut anastomoses are produced following intestinal surgery. The probable explanation for this lies either with the loss of competence of adjacent adult cell populations to respond to proximate growth and differentiation factors or with the cessation of secretion of these factors with the establishment of the adult phenotype. Competence may lie with the development of receptors, the synthesis of a missing link in a developmental pathway, or the repression of an inhibitor. Future work will explore the nature of cell–cell interactions in gut development using the chimaeric model described here.

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