Colonic Hamartoma Development by Anomalous Duplication in Cdx2 Knockout Mice

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ABSTRACT

To determine the biological role of caudal-like homeobox gene CDX2, we constructed knockout mice in which its mouse homologue Cdx2 was inactivated by homologous recombination, placing a bacterial lacZ gene under the control of the Cdx2 promoter. Although the homozygous mutants died in utero around implantation, the heterozygotes were viable and fertile and expressed lacZ in the caudal region in early embryos and in the gut tissues in adults. The heterozygotes developed cecal and colonic villi by anteriorization and formed hamartomatous polyps in the proximal colon. The hamartoma started to develop at 11.5 days of gestation as an out-pocket of the gut epithelium, which ceased to express the remaining Cdx2 allele. The out-pocket then expanded as a partially duplicated gut but was contained as a hamartoma after birth. In adult mice, these hamartomas grew slowly and took a benign course. None of them progressed into invasive adenomas or carcinomas, even at 1.5 years of age. Whereas the cecal and colonic villi expressed lacZ, the hamartoma epithelium did not, nor did it express Cdx2 mRNA from the wild-type allele. However, genomic DNA analysis of the polypl epithelium did not show a loss of heterozygosity of the Cdx2 gene, suggesting a mechanism of biallelic Cdx2 inactivation other than loss of heterozygosity. These results indicate that the Cdx2 haploinsufficiency caused cecal and colonic villi, whereas the biallelic inactivation of Cdx2 triggered anomalous duplications of the embryonic gut epithelium, which were contained as hamartomas after birth.

INTRODUCTION

The Drosophila caudal gene, the first member of the caudal-type homeobox gene family to be described, is expressed in a gradient pattern highest at the posterior pole of the embryo (1). Whereas the caudal mutants lose posterior structures (2), the ectopic expression of caudal leads to a disruption of head segmentation and development (3). Several other caudal-like genes have been isolated in mammals. These include Cdx1, Cdx2, and Cdx4 in the mouse, Cdx3 in the hamster (4), and CDX1 (5) and CDX2/3 (6) in the human. Expression of Cdx2 in the mouse embryo begins at 3.5 dpc (7) and is confined to the trophectoderm but absent from the inner cell mass (7). Subsequently, it is expressed in the extraembryonic ectoderm adjacent to the epiblast, sparing the more superficially placed polar and mural trophectodermal blast. Its expression continues in the fetal membranes, such as the chorion and allantoic bud, and in the spongiotrophoblasts at later stages. After 8.5 dpc, Cdx2 is expressed principally in the posterior gut, tail bud, and cecal neural tube (7).

The CDX2 protein has been characterized as a key transcriptional factor for intestinal development. It regulates some intestine-specific genes such as sucrase-isomaltase and carbolic anhydrase-1, which are indispensable for differentiation and homeostasis of the intestine (8). Cdx2 has been reported to play a role in the extracellular matrix-mediated intestinal cell differentiation (9). A gene knockout analysis showed that the homozygous null Cdx2 mutants die between 3.5 and 5.5 dpc, whereas the heterozygous mutant mice are fertile, with skeletal malformations, and form multiple adenomatous polyps in the colon (10). Although several reports suggest that expression of Cdx2 is down-regulated in human colon cancer cells (5, 11), it remains to be determined whether the down-regulation is responsible for colorectal carcinogenesis.

To investigate the expression and function of Cdx2, we have constructed ES cell lines in which the Cdx2 gene was inactivated by homologous recombination, with a lacZ cassette placed under the control of the Cdx2 promoter-enhancer. We have established Cdx2 mutant mice from these ES cells and analyzed Cdx2 expression and the precise mechanism of polyp formation.

MATERIALS AND METHODS

Gene Mapping of Cdx2. We used a panel of DNA samples from interspecific backcross mice that has been characterized for over 1100 genetic markers throughout the genome (12). The informative restriction fragment length variants for Cdx2 were defined using Mspl; the C3H allele showed a band of 1.8 kb, whereas that of Mus spretus was 2.3 kb.

Constitution of Cdx2 Targeting Vector. A recombinant A clone containing the mouse Cdx2 was isolated from a 129/Sv genomic library using a Cdx2 genomic DNA fragment probe [nucleotide positions 14–1153 according to James et al. (13)]. A 9.6-kb BgIII-NolI fragment, containing a part of exon 1, and another downstream fragment of about 900 bp (NolI-KpnI) were subcloned in pUC19, respectively. The Cdx2 ATG translation initiation codon in exon 1 was deleted and replaced with a cassette containing the lacZ reporter and PGK-neo+bp [Ref. 14; Fig. 1A].

Transfection of ES Cells and Selection of Targeted Clones. Fifty µg of the targeting vector were linearized at the unique Sse8387 site and electrophoresed into E5 cell line cell RW4 (Genome Systems, St. Louis, MO) as described (15). Of 200 G418-resistant colonies, 3 independent homologous recombinant candidates were identified by PCR and confirmed by Southern hybridization. The PCR primers were: Cdx2-F2, complementary to the neo gene (5′–CCG GTG GTG GAT GTG AAG TGA GTG-3′); and Cdx2-R2, located 75 bp downstream from the 3′ KpnI site in Cdx2 (5′–CCC CCT CAG TAG ACC CTA AT-3′); Fig. 1). Amplifications were performed for 35 cycles (denaturation, 1 min at 94°C; hybridization, 1 min at 53°C; and elongation, 2 min at 72°C) and the homologous recombinants showed a 973-bp band (Fig. 1B). Upon Southern analysis, their BamHI-digested genomic DNA showed two bands, 3.5 and 4.0 kb long, corresponding to the wild-type and knockout alleles, respectively (Fig. 1B). The probe used was a 630-bp PstI-XbaI fragment containing the neo′ or a 1246-bp fragment downstream of the 3′ KpnI site (Fig. 1A).

Generation and Genotyping of Mutant Mice. Two germ-line chimeras were obtained (Fig. 1D). The wild-type allele was identified by PCR using two primers: Cdx2-end (5′–TTT GTC AGT CCT CGG CAG TA-3′); and Cdx2-R2, as described above, except that annealing was at 55°C (Fig. 1C). Although most data presented here were collected in mice with the (129/Sv × C57BL/6J)F1 background, we also analyzed the mice backcrossed to C57BL/6 (N2–N4) and found the same phenotypes.

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3 The abbreviations used are: dpc, days post coitum; ES, embryonic stem; Xgal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; RT-PCR, reverse transcription-PCR; BrdUrd, bromodeoxyuridine; PJS, Peutz-Jeghers syndrome; Ck8, cytokeratin 8; LOH, loss of heterozygosity.
Fig. 1. Inactivation of the mouse Cdx2 gene by homologous recombination. Panel A, targeting strategy. Structures of the wild-type allele, targeting vector pBgk, and the knockout allele are shown. pBgk contained two regions of homology (LA and SA) to the genomic DNA, sandwiching a lacZ reporter-neo selection cassette. Homologous recombination resulted in the deletion of 75 bp from exon 1 (ex1), and the lacZ reporter was transcribed from the Cdx2 promoter. Filled boxes, exons (ex1, ex2, and ex3); solid lines, the noncoding regions. Pairs of arrowheads, positions of the PCR primers used for identification of the knockout and the wild-type alleles, respectively. Solid line, probe used for the Southern hybridization. B, BamHI site. Arrows, transcriptional directions for the respective marker genes. Panel B, Southern blot analysis of the wild-type (+/+ ) and heterozygous (+/- ) mouse tail DNA samples. The BamHI-digested fragments of 3.5 and 4.0 kb were derived from the wild-type (WT) and knockout alleles (KO), respectively. Panel C, transmission of the knockout allele to the progeny as determined by PCR: the knockout (KO; 973 bp) and wild-type (WT; 895 bp) alleles, respectively. Lane M contained size markers of 1.35, 1.08, 0.87, and 0.60 kb. Panel D, expression of Cdx2 mRNA analyzed by RT-PCR in the cultured blastocysts from an F1 heterozygous intercross. Arrowhead, the band for the amplified Cdx2 cDNA segment (1.0 kb). PC, positive control using the Cdx2 cDNA as the template. Lane M was loaded with size markers as in C.

Detection of β-Galactosidase Activity. The day of the vaginal plug was considered as 0.5 dpc. To detect the β-galactosidase activity, the samples were incubated at 30°C for 30 min to overnight, depending on the developmental stage, in a staining solution (5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 1 mg/ml X-gal in PBS).

In Situ Hybridization. Essentially, the same procedures were used as described previously (13), except that alkaline phosphatase-coupled antidigoxigenin antibody (Boehringer Mannheim, Mannheim, Germany) was used. Whole-mount embryos were hybridized and sectioned.

Culture of Cdx2 Blastocysts and Polyp Tissues. Embryos were flushed from uterus at 3.5 dpc, placed in gelatin-coated wells in HEPES-buffered DMEM supplemented with 20% fetal bovine serum, and incubated at 37°C in 5% CO2 in air. The polyp cells were cultured as described (16) with a minor modification.

RT-PCR. Total RNA from the cultured cells were reverse-transcribed to cDNA with random hexamer primers, and each transcript was detected by PCR using oligonucleotide primers: Cdx2 mRNA, 5'-GGC CAC CAT GTA CGT GAG CT-3' and 5'-GTC ACT GGG TGA CAG TGG AG-3' (13); lacZ mRNA, 5'-GCC TTA CCC AAC TTA ATC G-3' and 5'-TGT CAG CGA GTA ACA ACC-3'; and Ck8 mRNA, 5'-ACC ACC AGC GGC TAC TCA GGA GGA C-3' and 5'-CTT GGA CAC GAC ATC AGA AGA CTC G-3'. Thirty-five cycles were performed as described above, except that hybridization was at 53°C or 63°C.

BrdUrd Labeling. Incorporations of BrdUrd were for 4 h for adults and 1 h for embryos, as described (18).

RESULTS

Cdx2 Maps on Mouse Chromosome 5. It has been reported that mouse Cdx2 maps on chromosome 5 in an interspecific backcross mapping using a panel of (C57BL/6J × M. spretus)F1 × M. spretus mice (19). We independently mapped Cdx2 using different markers in another panel of 114 backcrossed mice from (C3H/HeJ-gld × M. spretus)F1 × C3H/HeJ-gld (12). The Cdx2 gene was found tightly linked to the myocyte nuclear factor (Mnf; Ref. 20) and platelet-derived growth factor α (Pdgfa; Ref. 20) genes on distal chromosome 5 and not very far from the β-glucuronidase (Gus; Ref. 21) or erythropoietin (Epo; Ref. 22) gene. The linkage distances and the gene order were: Gus - 1.8 ± 1.2 cM - Epo - 1.8 ± 1.2 cM - Cdx2/Mnf/ Pdgfa. These results are consistent with, and complementary to, those reported recently (19). No known mouse mutations were found in the vicinity of the Cdx2 locus that were likely to be Cdx2 mutant alleles.

Cdx2(-/-) Embryos Are Lethal. One of the Cdx2 alleles was inactivated by homologous recombination in ES cells so that the translation initiation site was disrupted by a bacterial β-galactosidase gene (lacZ; Fig. 1; see “Materials and Methods”). Their germ-line chimeras were generated, and the resultant heterozygotes were viable and fertile (see below), whereas no homozygous pups were obtained from the heterozygous intercrosses. Although all embryos genotyped from 7.5 to 13.5 dpc were either wild type or heterozygous at the ratio of ~1:2 (actual numbers, 17:38), the Mendelian ratio was obtained when the morulae and blastocysts were cultured in vitro for 7 days and genotyped (actual numbers, 4:8:4). However, the homozygous cells of the inner cell mass failed to proliferate, although the trophectoderm cells showed normal outgrowths (data not shown). As expected, an RT-PCR analysis of the embryonic cells thus cultured revealed that the homozygous mutants lacked the Cdx2 mRNA, whereas the wild-type and heterozygous embryos expressed the gene (Fig. 1D). These results suggest that the homozygous embryos failed to develop after implantation.

Cdx2(+/-) Mice Develop Cecal and Colonic Villi and Colonic Hamartomas. It has been reported that Cdx2 heterozygous mice show an anterior homeotic shift of vertebrae and corresponding malformations of the ribs and develop multiple intestinal adenomatous polyps, particularly in the proximal colon (10). We also confirmed the skeletal anomaly as well as their runted growth (data not shown). In addition, examination of the gut of the adult Cdx2 heterozygotes revealed abnormal structures in the cecum and the proximal colon. Whereas the mucosa of the wild-type cecum and colon consisted of crypts without villi (Fig. 2B), the homozygous mutants contained numerous villiform structures in the cecum and proximal colon (Fig. 2A, C, D, G, H, J, and K). The densities of these structures were lower than that of the villi in the ileum, and they were often aligned in rows (Fig. 2C). They were found abundantly in the body of the cecum, with decreasing densities and heights toward the apex (data not shown). Histologically, these villiform structures resembled the villi in the ileum but were extended from crypts of the colonic type, with their epithelium containing enterocytes and a few goblet cells (Fig. 2D). Fine blood vessels were evident in the villiform structures, and the lacteals appeared to exist. Immunohistochemistry with antibodies against the small intestine-specific peptide transporter and sucrase-isomaltase, respectively, showed expression of these proteins in the structures (data not shown). Accordingly, they can be explained as the result of an incomplete anteriorization of the cecal and colonic epithelia caused by the haploinsufficiency of the Cdx2 gene expression (see below).

In addition to the villiform structures above, some polyps were found in the proximal colon. In adult mice, they had a sessile or pedunculate configuration, ranging from one-half to 8 mm in diameter. At 3 months of age, up to 10 polyps were found per mouse. Most
colonic hamartomas in the adult mice did not contain any dysplastic changes such as prominent budding, crypt branching or resultant crowding/expansion of the epithelium, or cytological atypia as hyperchromatism or nuclear stratification. To determine the nature of the polyp cells further, we labeled nascent DNA with BrdUrd and stained the sections with a specific antibody. Whereas parts of the polyp epithelium incorporated some BrdUrd, as shown in Fig. 3E, the center of the hamartoma incorporated BrdUrd relatively little (Fig. 3F). These results are consistent with a benign course of the polyps. Although all heterozygotes carried polyps, the life spans of the heterozygotes were the same as their wild-type littermates, at least up to 1.5 years of age. The polyps in such mice did not show any signs of malignant changes (data not shown).

To monitor the Cdx2 expression in the heterozygotes, we stained their intestinal tissues with X-gal for the lacZ reporter activity. In the ileum, the villi were stained more intensely than the crypts, although the Paneth cells at the base were not stained (Fig. 4A). In contrast, the hamartoma tissues were not stained with X-gal, despite the fact that the villiform structures nearby were stained (Fig. 4B). To characterize this phenomenon further, we determined the expression of the Cdx2 and lacZ mRNAs by RT-PCR analyses. Because it was difficult to separate the polyp epithelial cells from the stromal cells, we cultured the polyp tissue in vitro and established a polyp epithelium-derived cell population, which aggregated as colonies (Fig. 4C). Neither transcript of the lacZ nor Cdx2 gene was detected (Fig. 4D), while the cells expressed the cytokeratin 8 (Krt2–8) mRNA, verifying their epithelial origin (Fig. 4D). Thus, we conclude that both Cdx2 alleles were inactivated in the hamartomas. To investigate whether the tumor cells lost the wild type Cdx2 allele (i.e., LOH), a PCR genotyping was then carried out for each colony. In all colonies that showed biallelic inactivation of Cdx2, both the wild-type and knockout Cdx2 gene
Cdx2 does not appear to be the mechanism of its inactivation in the polyps in situ. Additional hamartoma-derived cells, whereas lac control cDNA for each gene. Note that neither sis. Of four independent polyps were amplified by PCR followed by agarose gel electrophoresis. hamartoma cells.

Arrowheads

Fig. 4. Molecular analysis of the hamartoma in the Cdx2 heterozygotes. A, section of the ileum prestained for the lacZ activity (H&E staining). Note a marked lacZ expression in the goblet cells (arrowheads). B, frozen section of a polyp in the proximal colon stained for the lacZ activity. Note that the hamartoma is not stained (arrowhead). In contrast, the villiform structures surrounding the hamartoma show a significant staining (arrow). Bar, 100 μm. C, phase-contrast micrograph of the hamartoma cells cultured in vitro. A focal growth of the epithelial cells is observed on the feeder layer (arrow). Bar, 100 μm. D, RT-PCR analysis for the expression of lacZ, Cdx2, and Krt2–8 (Ck8) in cultured hamartoma cells. Lanes 1–4, cells derived from four independent polyps; PC, positive control cDNA for each gene. Note that neither lacZ nor Cdx2 was expressed in the hamartoma-derived cells, whereas Ck8 was expressed in all clones. E, analysis of LOH in the Cdx2 gene in cultured hamartoma cells. DNA samples isolated from the cultured cells of four independent polyps were amplified by PCR followed by agarose gel electrophoresis. Lanes −, PCR products from the knockout allele; Lanes +, wild-type allele. Arrowheads, band positions for the knockout allele (973 bp; black) and the wild-type allele (895 bp; white), respectively. Lane M, size makers (dx174 RF DNA digested with HaellII).

DISCUSSION

Compared with an earlier report of Cdx2 knockout mice (10), we found some distinctly different gastrointestinal phenotypes, i.e., the heterozygotes developed cecal and colonic villiform structures and colonic hamartomas. It is likely that the villiform structures resulted from a homeotic anterior transformation of the gut mucosa due to the Cdx2 haploinsufficiency, because Cdx2 transcripts are abundant in the normal cecum and proximal colon (13), and expression of Cdx2 monitored by lacZ continued in the gut from the embryonic to adult life. On the other hand, the hamartomas appeared to be caused by the biallelic Cdx2 inactivation, because their epithelial cells did not express either lacZ or the remaining Cdx2 allele (Figs. 4 and 5).

In the Apc knockout mice, the crypt proliferative zone cells form outpockets after birth, beginning about 3 weeks of age (15, 18). In the Cdx2 knockout mutant, however, the outpocket formation took place around 11.5 dpc, when the epithelium differentiation was suppressed by the biallelic inactivation of Cdx2, which probably kept the cells proliferating faster than the surrounding epithelium. Forced expression of Cdx2, on the other hand, results in differentiation and slower growth rates in rat intestinal epithelial cell line IEC-6 (25).

The outpocketed epithelium developed thereafter into a partially duplicated gut structure (Fig. 5f). However, this duplication is different from those often seen in teratological anomalies of the human gastrointestinal tracts. Such duplications are suggested to form by errors of recanalization after the stage of complete occlusion of the intestinal lumen (26) or by errors of separation between the notochord and embryonic endoderm (27). In the Cdx2 hamartomas, on the other hand, the initially duplicated gut epithelium did not go through any occluded stages, nor did it continue the normal development after 16 dpc; it was outgrown by the normal gut and contained as a hamartoma
Fig. 5. Hamartoma formation in Cdx2 heterozygotes during gut differentiation. Dissected embryonic guts stained for the β-galactosidase (lacZ) activity (A–M, except E, I, J, and L) or hybridized in situ for Cdx2 mRNA (E and I) were analyzed. Sections of the proximal colon from the mice after birth were stained with H&E (N–Q). A, gut dissected from a 10.5 dpc embryo together with the tail bud (†). B, LacZ expression in the simple epithelium (e) of the proximal colon (10.5 dpc). C, outpocket (p) in the proximal colon formed opposite to the cecal bud (c; 11.5 dpc). D, cross-section through the outpocket (p) on the mesenteric (m) side expressing lacZ (11.5 dpc). E, in situ hybridization for Cdx2 mRNA at 11.5 dpc, showing lack of its expression in the outpocket (p), even at its base. F, disappearance of the lacZ expression from the tip of the outpocket (p) at 12.0 dpc. G, formation of the cecum (c) and disappearance of lacZ expression from the outpocket (at p, not visible; i, ileum; 12.5 dpc; see H and I). H, section showing an outpocket (p) lacking lacZ expression. I, in situ staining for Cdx2 mRNA at 12.5 dpc, showing lack of Cdx2 expression in the outpocketed gut epithelium (p). J, BrdUrd labeling of nascent DNA in the gut epithelium at 12.5 dpc. Note more labeling (arrows) in the outpocketed epithelium (p). K, section of the proximal colon at 15.5 dpc illustrated in L. L, schematic drawing of the relationship between the gut and the duplicated structure at 15.5 dpc (see K). Note that lacZ was not expressed in the duplicated structure (d). M, section showing an opening (o) of the duplicated structure (17.5 dpc). Note that the colonic epithelium was forming crypts and expressed lacZ, whereas that of the duplicated structure did not form crypts or express lacZ. N, histological section of the proximal colon at 4 days after birth (H&E staining). Note the pseudostratified epithelium (s) in the originally duplicated structure, which was connected to the normal epithelium of the colon at the opening (o). O, histological section of the proximal colon at 1 week after birth, showing the pseudostratified epithelium (s) and its surrounding mesenchymal walls (w). P and Q, serial sections of the proximal colon at 2 weeks after birth. Note the originally duplicated structure being contained as a hamartoma. The pseudostratified epithelium (s) was located underneath the opening of the polyp (o). Also note the branching ducts in the polyp (+ in P) and the thin wall (t) about to contain the originally duplicated structure. Bars: B, D, and E, 50 μm; F–Q (except for L), 100 μm. c, cecum; d, duplicated structure; e, simple or columnar epithelium; i, ileum; m, mesentery; o, opening of the duplicated structure; p, outpocket; s, pseudostratified epithelium; t, thin wall; w, mesenchymal wall.
after birth. Hamartomas are nonneoplastic nodules composed of an abnormal overgrowth of indigenous cell types with evidence of an underlying developmental etiology (28). Most human intestinal hamartomas are found along the mesentery (23). Interestingly, the outpockets extruded into the mesenchyme along the mesenteric arteries. In the adult CdX2 heterozygotes, the hamartomas were also found along the mesentry of the proximal colon.

It is worth noting that transgenic mice expressing Hox3.1 (HoxC8) from the Hox1.4 (HoxA4) cis-regulatory elements develop hamartoma
tous nodules in the stomach in addition to homeotic transformations of the skeletal system (29). Recently, several genes have been identified, the mutations of which are responsible for inherited hamartoma polyposis syndromes such as Cowden syndrome, Bannayan-Ruvalcaba-Riley syn


drome, and PJS (30). Although Cowden syndrome and Bannayan-Ruvalcaba-Riley syndrome appear to be caused by mutations in PTEN/ MMAC1/TEP1, a gene encoding phosphatidylinositol (3, 4, 5) triphos
phate, phosphatase (31), PJS is caused by germ-line mutations in a protein serine-threonine kinase gene LKB1/STK11 (32). Thus far, there have been no hereditary hamartoma syndromes reported that carry muta
tions in the Hox or CdX2 gene. It is interesting that Hox3.1 (HoxC8) is expressed in the mesenchyme of the stomach, whereas CdX2 is expressed in the epithelium. The histological characteristics of the hamartomas in the CdX2 heterozygotes are most similar to those of PJS, and PJS polyps can have features of diverticula, suggesting a common mode of formation. However, there were distinct differences as well, such as the lack of goblet cells and the presence of the pseudostratified epithelium that can have features of diverticula, suggesting a common mode of forma

tion. However, there were distinct differences as well, such as the lack of goblet cells and the presence of the pseudostratified epithelium that showed keratinization. Because the latter was seen only after birth, it is possible that the homeostatic changes upon milk digestion may be re-


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