Key Role of the Cdx2 Homeobox Gene in Extracellular Matrix–mediated Intestinal Cell Differentiation

Olivier Lorentz, Isabelle Duluc, Adèle De Arcangelis, Patricia Simon-Assmann, Michèle Kedinger, and Jean-Noël Freund

Institut National de la Santé et de la Recherche Médicale, Unité 381, 67200 Strasbourg, France

Abstract. To explore the role of homeobox genes in the intestine, the human colon adenocarcinoma cell line Caco2-TC7 has been stably transfected with plasmids synthesizing Cdx1 and Cdx2 sense and antisense RNAs. Cdx1 overexpression or inhibition by antisense RNA does not markedly modify the cell differentiation markers analyzed in this study. In contrast, Cdx2 overexpression stimulates two typical markers of enterocytic differentiation: sucrase-isomaltase and lactase. Cells in which the endogenous expression of Cdx2 is reduced by antisense RNA attach poorly to the substratum. Conversely, Cdx2 overexpression modifies the expression of molecules involved in cell–cell and cell–substratum interactions and in transduction process: indeed, E-cadherin, integrin-β4 subunit, laminin-γ2 chain, hemidesmosomal protein, APC, and α-actinin are upregulated. Interestingly, most of these molecules are preferentially expressed in vivo in the differentiated villi enterocytes rather than in crypt cells. Cdx2 overexpression also results in the stimulation of HoxA-9 mRNA expression, an homeobox gene selectively expressed in the colon. In contrast, Cdx2-overexpressing cells display a decline of Cdx1 mRNA, which is mostly found in vivo in crypt cells. When implanted in nude mice, Cdx2-overexpressing cells produce larger tumors than control cells, and form glandular and villus-like structures.

Laminin-1 is known to stimulate intestinal cell differentiation in vitro. In the present study, we demonstrate that the differentiating effect of laminin-1 coatings on Caco2-TC7 cells is accompanied by an upregulation of Cdx2. To further document this observation, we analyzed a series of Caco2 clones in which the production of laminin-α1 chain is differentially inhibited by antisense RNA. We found a positive correlation between the level of Cdx2 expression, that of endogenous laminin-α1 chain mRNA and that of sucrase-isomaltase expression in these cell lines.

Taken together, these results suggest (a) that Cdx1 and Cdx2 homeobox genes play distinct roles in the intestinal epithelium, (b) that Cdx2 provokes pleiotropic effects triggering cells towards the phenotype of differentiated villus enterocytes, and (c) that Cdx2 expression is modulated by basement membrane components. Hence, we conclude that Cdx2 plays a key role in the extracellular matrix–mediated intestinal cell differentiation.

Homeobox genes encode nuclear transcription factors involved in patterning and cell differentiation during development of metazoans (McGinnis and Krumlauf, 1992). They have also been identified as a new class of protooncogenes (Maulbecker and Gruss, 1993), and substantial evidences indicate that homeobox gene alterations participate in tumor genesis (see Cillo, 1994). Considerable progress in understanding the function of homeobox genes arose from the finding that they regulate molecules involved in cellular interactions such as cell adhesion molecules and extracellular matrix components (Edelman and Jones, 1993). Homeobox genes themselves are regulated by other homeobox genes (Hayashi and Scott, 1990), retinoids (Mavilio, 1993; Gudas, 1994), and/or growth factors (Ruiz i Altaba and Melton, 1989; Pavlova et al., 1994).

Homeobox genes of the caudal family, Cdx1 and Cdx2, are expressed in the intestinal epithelium (James and Kazenwadel, 1991). In Drosophila, caudal is involved in anterior-posterior patterning (Macdonald and Struhl, 1986; Mlodzik and Gehring, 1987). In mammals, Cdx1 was the first homeobox gene discovered in endodermal tissues (Duprey et al., 1988; Hu et al., 1993); it exhibits an increasing gradient expression along the longitudinal axis of gut (James and Kazenwadel, 1991; Freund et al., 1992), and the...
protein is predominantly expressed in undifferentiated crypt cells (Silberg et al., 1997). Cdx2, another caudal-related gene present in the intestine, encodes a nuclear transcriptional factor that is expressed in the differentiated enterocytes (James et al., 1994) and binds cis-elements present in the gene promoters of enterocyte markers such as sucrase-isomaltase (SI)\(^1\), lactase-phlorizin hydrolase (LPH), apolipoprotein B, carbonic anhydrase 1, and calbindin D9K (Suh et al., 1994; Drummond et al., 1996; Lambert et al., 1996; Lee et al., 1996; Troelsen et al., 1997). An important role has been attributed to Cdx2 in intestinal cell differentiation because this homeobox gene triggers SI expression and cell polarization in undifferentiated intestinal IEC cells in vitro (Suh et al., 1994; Suh and Traber, 1996). Furthermore, using xenograft models in which the cell fate of the fetal intestinal endoderm can be modulated by its association with mesenchymal cells of different origins (Duluc et al., 1994; Fritsch et al., 1997), we have observed a correlation between the level of Cdx2 (and Cdx1) expression, and the small-intestinal–like versus colonic-like morphogenesis and differentiation of the grafted epithelial cells (Duluc et al., 1997). These observations suggest that homeobox genes of the caudal family participate in intestinal differentiation and that their expression is dependent on epithelial/connective tissue interactions.

The involvement of epithelial–mesenchymal cell interactions in the control of cell differentiation during intestinal ontogeny and during the continuous cell renewal in the mature organ has been demonstrated (Simon-Assmann and Kedinger, 1993; Simon-Assmann et al., 1995). Evidence of the functional role played by reciprocal cellular interactions was provided by grafting experiments of various tissue recombinants (Haffen et al., 1989; Kedinger et al., 1990; Duluc et al., 1994). The basement membrane at the interface between epithelial and mesenchymal cells participates in these interactions (Simon-Assmann et al., 1995). Of particular interest is the finding that laminin-1, consisting of α1/β1/γ1 chains and synthesized by both epithelial and mesenchymal cells (Simo et al., 1991, 1992a), promotes intestinal cell differentiation in vitro (Hahn et al., 1990; Vachon and Beaulieu, 1995; Basson et al., 1996). In addition, cells grown in the presence of anti-laminin-1 antibodies or cells in which the endogenous production of laminin-1 is inhibited by antisense RNA, fail to complete their differentiation, as assessed by the absence of enterocytic markers such as LPH and SI (Simo et al., 1992b; De Arcangelis et al., 1996).

In an attempt to approach the role of caudal-type homeobox genes in the intestinal epithelial cell behavior and in the response to the basement membrane, we have analyzed (a) the phenotypic changes resulting from the modification in the level of Cdx1 or Cdx2 expression in a human colonic adenocarcinoma cell line (Caco-2/TC7; Chantret et al., 1994), and (b) the level of Cdx2 expression in Caco2 cells cultured on exogenous laminin substratum or producing lamin-α1 chain antisense RNA.

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\(^1\) Abbreviations used in this paper: EHS, Engelbreth-Holms-Swarm; FAK, focal adhesion kinase; HD1, component of hemidesmosome; LPH, lactase-phlorizin hydrolase; RT-PCR, reverse transcriptase-polymerase chain reaction; SI, sucrase-isomaltase.

**Materials and Methods**

**Cells and Injections in Nude Mice**

The Caco2-derived cell clone TC7 (Chantret et al., 1994) was grown under 5% CO\(_2\), 95% air atmosphere in DME (GIBCO BRL, Courbevoie, France) containing 20% fetal calf serum, 1% nonessential amino acids, 1% penicillin, 1% streptomycin, and 0.1% gentamicin. Cells seeded at low density (1.2 × 10\(^4\) cells/cm\(^2\)) were passaged every 7 d. For some experiments, they were cultured on coatings of laminin-1 (10 μg/cm\(^2\)) extracted from Engelbreth-Holms-Swarm (EHS) tumors, as described by Simon-Assmann et al. (1994a). Caco2 clones transfected with the plasmid pCB6/AS-LN producing laminin-α1 chain antisense RNA (De Arcangelis et al., 1996) were grown in the presence of 1.2 or 0.6 μg/ml G418 (geneticin; GIBCO BRL). RNA extraction, protein preparation, and immunocytochemical experiments were performed on cells grown for 5 d.

For grafting experiments, 10\(^5\) cells were injected subcutaneously to nude mice and the tumors were recovered 7 or 12 wk after injection.

**Adhesion and Aggregation Tests**

Adhesion and aggregation tests were performed with transfected TC7 cell clones. Adhesion tests were performed as described previously (Simon-Assmann et al., 1994a) on microtiter wells coated or not with 1 μg/well EHS-extracted laminin-1 followed by an incubation with 1% bovine serum albumine (V/H fraction; Sigma, St. Quentin Fallavier, France). In inhibition studies with antiintegrin antibodies (Table I), the cell suspension was mixed with antiintegrin-β1 (1/8,100 final dilution), antiintegrin-β4 (1/250 final dilution) or antiintegrin-α6 (1/8,100 final dilution) antibodies before plating on the coated wells (Orian-Rousseau, V., personal communication). Changes in cellularity after 1 and 2 h were measured by the binding of crystal violet dye at pH 5.5 to fixed cultures at an optical density of 595 nm.

Aggregation tests were carried out according to the procedure described by Berndorff et al. (1994). Briefly, after dissociation, 3 × 10\(^5\) cells were placed into 5 ml DME medium and agitated at 25°C; the number of single cells and small aggregates up to four cells were counted every 10 min. Aggregation was scored according to the decrease in the percent of isolated cells compared to those present in the suspension at the beginning of the experiment.

**RNA Extraction, Northern Blots**

RNA was extracted from cultured cells and tumors using Trizol Reagent (GIBCO BRL) according to the recommendations of the supplier. After cell homogenization, the mixture was centrifuged for 5 min at 12,000 g to discard genomic DNA contained in the pellet. RNA was treated at 37°C for 30 min with 0.25 U RNase-free DNase/μg RNA (GenHunter, Nashville, TN). RNA concentration was determined by optical densitometry at 260 nm.

Northern blot analysis was performed under standard condition, as previously described (Duluc et al., 1994), using 32P-labeled probes (RediPrime kit; Amersham, Les Ulis, France).

**Reverse Transcriptase–Polymerase Chain Reaction (RT-PCR)**

Oligonucleotides were from Eurogentec (Seraing, Belgium). They derived from published genomic or cDNA sequences deposited in the GenBank/EMBL/DDBJ databank. Table II lists the oligonucleotides and the corresponding accession numbers in the databank.

Single-stranded cDNA was synthesized for one h at 42°C in 50 μl containing 6 μg RNA, 100 pmoles oligo-dT, 15 μL AMV reverse transcriptase (Promega Corp., Madison, WI), 0.4 mM each dNTP, 50 mM Tris-HCl, pH 8.3, 50 mM KCl, 10 mM MgCl\(_2\), 10 mM DTT, 0.5 mM spermidine, 4 mM sodium pyrophosphate. To specifically amplify endogenous Cdx1 mRNA in cells producing Cdx1 antisense RNA, cDNA synthesis was primed with 100 pmoles of the Cdx1b primer. PCR reactions (100 μl) contained 2 μl of cDNA solution, 50 pmol primers, 0.5 U Goldstar DNA polymerase (Eurogentec), 75 mM Tris-HCl, pH 9, 20 mM ammonium sulfate, 0.01% Tween 20, and 1.25 mM MgCl\(_2\). Dynaxaw (Eurogentec) was used to separate the template cDNA and primers from the DNA polymerase during pipetting and before the first step of denaturation. PCR used a Thermojet apparatus (Eurogentec). Conditions were: 94°C, 30 s; 55°C, 45 s; 72°C, 45 s. For each pair of primers, reaction was performed for 16 to 38 cycles to overlap the range of cycles in which the amount of PCR product increased.
exponentially. As internal standard, RT-PCR was carried out with primers hybridizing to the mRNA encoding the 36B4 ribosomal protein. Control PCR was performed directly on RNA without the step of cDNA synthesis; no amplified DNA fragment was detected in this case. In addition, primers used to visualize Cdx1, Cdx2, HoxC-8, and LPH transcripts were chosen in different exons; no PCR product corresponding to the genomic fragments was obtained, demonstrating the absence of contaminating genomic DNA in the RNA samples.

PCR fragments were loaded on 3% agarose gels and analyzed using an Imaging Densitometer (Bio-Rad Laboratories, Hercules, CA). RT-PCR fragments were inserted in the pGEM-T plasmid (Promega Corp.) and sequenced using the T7 DNA polymerase sequencing kit (Pharmacia Diagnostics AB, Uppsala, Sweden) to confirm their identity.

Construction of Recombinant Expression Plasmids

General procedures to construct recombinant plasmids were as described by Sambrook et al. (1989). Cdx1 and Cdx2 cDNA fragments were inserted in the PCR expression vectors (Brewer, 1994) that contains the cytomegalovirus promoter and the 3′-untranslated region and polyadenylation signal of the human growth hormone (a generous gift of Dr. G. Perrozzi, INN, Rome, Italy).

The complete open reading frame of the murine Cdx1 gene was reconstituted using pbH8 that contains a nearly full-length cDNA (Duprey et al., 1998), and pCxD1-34 that contains the 4-kb Small restriction fragment overlapping the 5′ region of the murine Cdx1 gene (kindly provided by Dr. P. Duprey, Université Paris VII, Paris, France). The 1.1-kb EcoRI-BamHI fragment of pCxD1-34, comprising the Cdx1 transcription start site and 0.3 kb of the transcription unit, was inserted in pUC19 (Pharmacia Diagnostics AB), and the resulting plasmid was cut with SphI to delete the upstream untranscribed segment. The NaeI-KpnI restriction fragment, and 0.3 kb of the transcription unit, was inserted in pUC19 (Pharmacia Diagnostics AB), and the resulting plasmid was cut with SphI to delete the upstream untranscribed segment. The NaeI-KpnI restriction fragment, and 0.3 kb of the transcription unit, was inserted in pUC19 (Pharmacia Diagnostics AB), and the resulting plasmid was cut with SphI to delete the upstream untranscribed segment. The NaeI-KpnI restriction fragment, and 0.3 kb of the transcription unit, was inserted in pUC19 (Pharmacia Diagnostics AB), and the resulting plasmid was cut with SphI to delete the upstream untranscribed segment.

Cell Transfections

TC7 cells were transfected with the plasmids pCdx1-S, pCdx1-AS, pCdx2-S, and pCdx2-AS, or with the control pCB6 vector using DOTAP-Reagent (Boehringer Mannheim, Mannheim, Germany). Briefly, cells were seeded at 50% confluency in 10-cm culture dishes. The next day, standard culture medium was replaced by 3-mL medium containing 3.5 µg of plasmid previously cut with the ScI1 restriction enzyme, dissolved in 25 µl containing 20 mM Hepes, pH 7.3, and 150 mM NaCl, and incubated 15 min at room temperature with 15 µg DOTAP-Reagent. After 18 h at 37°C, selection was started by adding 1.1 mg/ml G418 to the culture medium. 20 d later, several clones were picked out by capillary duct aspiration and propagated individually. The remaining G418-resistant cell populations from each culture dish were pooled.

Immunocytochemistry and Histology

Immunocytochemistry was performed on cells grown on coverslips for 5 d, or on 5 µm tumor cryosections. Cells were washed with PBS, fixed with 1% paraformaldehyde for 10 min, and permeabilized with 1% Triton X-100 for 10 min. To detect a component of hemidesmosome (HD1) protein, cells were fixed and permeabilized with methanol at -20°C for 5 min. Tumors were embedded in Tissue-Tek (Labonord, Villeneuve d’Asq, France) and frozen with isopentane cooled in liquid nitrogen. Cells and cryosections were incubated for 1 h with the primary antibodies (Table I) in PBS, and subsequently incubated with appropriate secondary antibodies conjugated with fluorescent isothiocyanate or Texas red (Pasteur Diagnostics, Paris, France; Interchim, Asnières, France; Amersham). Primary antibodies were omitted in controls. Cells or slides were mounted in glycerol/PBS/phénylenediamine, and observed with an Axiophot microscope (Zeiss, Oberkochen, Germany).

Morphological analysis was performed on tissues fixed in Bouin’s solution, embedded in paraffin, and stained with hematoxylin-cosin or periodic acid-Schiff.

Protein Preparations, Western Blots

Cell membrane proteins were prepared as previously described (Simon-Assmann et al., 1994a) to analyze the membrane distribution of integrin-α6 and -β4 subunits, and according to Fabre and de Herreros (1993) to analyze E-cadherin. CDX2 protein was detected in nuclear extracts prepared as described by Bertrand et al. (1995) with slight modifications. Cells were harvested, resuspended in 10 mM Hepes/KOH, pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 0.1% Triton X-100, and 0.2 mM phenylmethylsulfonyl fluoride, and homogenized with a tight-fitting Dounce homogenizer. Lysates were maintained 10 min on ice and centrifuged at 2,000 g for 10 min. The supernatant containing cytoplasmic and membrane proteins was immediately stored frozen at -70°C and further used to analyze the cellular level of APC, focal adhesion kinase (FAK), integrin-α6 subunit, integrin-β4 subunit, and α-actinin. The nuclear pellet was washed in 10 mM Hepes/KOH, pH 7.9, 1.5 mM MgCl2, 10 mM KCl, and 0.2 mM phenylmethylsul-
forny fluoride, and resuspended in 20 mM Hepes/KOH, pH 7.9, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 20% glycerol, and 0.2 mM phenylmethylsulfonyl fluoride. After 30 min at 4°C under constant agitation, nuclear debris were centrifuged at 13,000 g for 5 min and the supernatant was frozen and stored at −70°C before analysis of CDX2 protein content.

Protein samples (50 μg) were incubated at 100°C for 5 min in Laemmli buffer containing 2% SDS and 100 mM DTT. For immunoblot of integrin-β and integrin-β1 subunits, DTT was omitted. Proteins were separated by SDS-PAGE and transferred overnight to nitrocellulose filters by electroblotting in 25 mM Tris-HCl, 192 mM glycine, pH 8.2, and 20% methanol. Filters were saturated for 1 h at 37°C in PBS containing 5% skimmed milk. For immunodetection, filters were incubated 4 h at room temperature with appropriate primary antibodies (Table I) in PBS containing 1% skimmed milk and 0.1% Tween 20, and subsequently with secondary anti–mouse, anti–rabbit, or anti–rat antibodies labeled by HRP (Amersham Buchler GmbH, Braunschweig, Germany; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Detection by chemiluminescence was performed using Western blotting detection reagents (Amersham Buchler GmbH).

### Measurements of Lactase and Sucrase Activities

Lactase (EC 3.2.1.23) and sucrase (EC 3.2.1.48) activities were determined in brush border proteins according to standard protocols (Simon et al., 1979). The results were expressed as specific activities (mU/mg protein).

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**Table II. Synthetic Oligonucleotides**

| Gene | Name | Sequence | PCR product size | Accession number |
|------|------|----------|------------------|-----------------|
| Cdx1 | Cdx1a | ggcacctggcagcgcccctacgat | 252 | M80463 |
| Cdx1b | ggcacctggcagcgcccctacgat | 252 | M80463 |
| Cdx1c | ggcacctggcagcgcccctacgat | 252 | M80463 |
| Cdx1d | ggcacctggcagcgcccctacgat | 252 | M80463 |

Note: The table continues with similar information for other genes and sequences.
Transgene expression in transfected TC7 cells: expression of Cdx1 sense RNA in pCdx1-S-transfected cells (lane 1), of Cdx1 antisense RNA in pCdx1-AS-transfected cells (lane 2) and of Cdx2 sense RNA in pCdx2-S-transfected cells (lane 3). RT-PCR was performed on oligo-dT–primed cDNA for 24 cycles (lane 1) or 32 cycles (lane 2) with the oligonucleotide pairs Cdx1a/b and Cdx2b/c, respectively. (B) RT-PCR was made for 32 cycles with the oligonucleotide pair Cdx1a/b on cDNA primed with Cdx1b. The use of Cdx1b as primer for cDNA synthesis allows the specific detection of endogenous Cdx1 mRNA instead of the transcript synthesized from the recombinant plasmid. The lower panels in B and C show standard RT-PCR conducted for 22 cycles with primers designed to detect the 36B4 mRNA.

Results

Transfection of TC7 Cells with Plasmids Expressing Cdx1 and Cdx2 Sense and Antisense RNAs

The human colon adenocarcinoma cell line TC7 corresponds to a spontaneously differentiating clone derived from the original Caco2 cell population (Chantret et al., 1994). 5 d after seeding, they expressed a low level of Cdx1 and Cdx2 mRNA: RT-PCR fragments were observed after 32 amplification cycles, but could not be detected by 24 cycles (Fig. 1 A).

TC7 cells were transfected with plasmids synthesizing Cdx1 and Cdx2 RNAs in sense (pCdx1-S and pCdx2-S) and antisense orientations (pCdx1-AS and pCdx2-AS). Controls were transfected with the pCB6 vector. G418-resistant cell populations were obtained with every recombinant plasmid except with pCdx2-AS. In this case, G418-resistant cells grew after transfection but were lost during the first subculture because they no longer spread onto the culture dish after trypsin treatment. Transgene expression in the pooled G418-resistant cell populations was analyzed by RT-PCR using appropriate murine-specific primers (Fig. 1 B): Cdx1 sense and antisense RNAs, and Cdx2 sense RNA were already detected at 24 cycles indicating a high level of transgene expression in transfected cells. The ability of Cdx1 antisense RNA (produced in pCdx1-AS-transfected cells) to downregulate endogenous Cdx1 mRNA expression was investigated by RT-PCR on cDNA primed with the Cdx1b primer. The amount of Cdx1 mRNA was indeed lower in pCdx1-AS-transfected cells than in controls transfected with pCB6 (Fig. 1 C).

Growth and Differentiation of Cells Transfected with pCdx1-S, pCdx1-AS, and pCdx2-S

Growth and differentiation were analyzed in pooled cell populations transfected with pCdx1-S, pCdx1-AS, and pCdx2-S (Fig. 2). Compared to cells transfected with pCB6, onset of proliferation was delayed in the three cell populations, corroborating observations previously reported in Cdx2-overexpressing IEC cells (Suh and Traber, 1996). The cell number at the plateau was significantly lower in pCdx1-AS– and pCdx2-S–transfected populations than in controls, unlike pCdx1-S–transfected cells (Fig. 2 A). Sucrase and lactase activities, two markers of intestinal cell differentiation, were neither modified in pCdx1-S–transfected cells that overexpressed Cdx1, nor in pCdx1-
AS–transfected cells in which the endogenous level of Cdx1 mRNA was reduced (Fig. 2 B). In contrast, overexpression of Cdx2 in pCdx2-S–transfected cells caused a 9-fold and 1.7-fold increase of sucrase and lactase activities, respectively (Fig. 2 B). The corresponding mRNAs changed concomitantly (Fig. 2 C). This result is consistent with the fact that Cdx2 encodes a transfactor that binds to the SI and LPH gene promoters (Suh et al., 1994; Troelsen et al., 1997).

During standard subcultures, we noted that pCdx2-S–transfected cells at confluence exhibited high resistance to detachment with trypsin. Indeed, a 20 min treatment with 0.01% trypsin at 37°C was required to dissociate efficiently pCdx2–transfected cells instead of 5 min for cells transfected with pCB6, pCdx1-S, or pCdx1-AS. This suggests that modifications of cell–substratum and/or cell–cell interactions occurred in Cdx2-overexpressing cells.

To obtain further insight into the phenotypic and molecular changes induced by Cdx2 overexpression, cellular clones transfected with pCdx2-S were isolated by capillary duct aspiration and propagated individually. The selected C2S clone presented all the phenotypic changes described above as far as transgene expression, growth curve, resistance to trypsin, and brush border enzyme activities are concerned. In the following studies, the C2S clone has been compared to CB6, a clone transfected with the control plasmid pCB6. Western blotting performed using the polyclonal antiserum raised against the pancreatic CDX3 protein known to be identical with intestinal CDX2 (German et al., 1992; Laser et al., 1996) demonstrated a higher level of CDX2 in nuclear extracts of C2S cells compared to CB6 (Fig. 3 A). C2S cells also displayed a 2.2- and 18-fold higher lactase and sucrase activity than CB6 cells (data not shown).

Expression of Homeobox Genes in C2S Cells

In Drosophila (Dearolf et al., 1989) and more recently in mice (Subramanian et al., 1995), genes of the caudal family have been shown to control the expression of other homeobox genes. We have compared the expression of HoxA-4, HoxA-9, HoxC-8, and Cdx1 in C2S and in control CB6 cells because these genes exhibit specific and distinct patterns of expression along the anterior-posterior axis of gut (James and Kazenwadel, 1991; Freund et al., 1992; Duluc et al., 1997). Fig. 3 B indicates that HoxA-4 and HoxC-8 expression was similar in both cell lines; in contrast, the level of Cdx1 mRNA was markedly reduced in C2S cells whereas HoxA-9 was upregulated in these cells compared to CB6. Similar variations were observed between the pooled populations of cells transfected with pCdx2-S and pCB6 (not shown). We have also analyzed HNF-1α mRNA expression because this nuclear factor, like CDX2, binds to the sucrase-isomaltase gene promoter (Wu et al., 1994). HNF-1α mRNA was present, though unchanged, in both cell types (Fig. 3 B).

Expression of Molecules Involved in Cell–Cell and Cell–Substratum Interactions

Because several genes encoding cell adhesion molecules are the targets of homeoprotein regulation (Edelman and Jones, 1993), and because Cdx2-overexpression cells display a high resistance to trypsin treatment at confluence, we have compared the expression of a panel of molecules involved in cell–cell and cell–substratum interactions in C2S and CB6 cells. These molecules have been analyzed at the protein and mRNA levels by immunocytochemistry, Western blotting, and/or RT-PCR.

Analysis of Proteins Belonging to the Cadherin–Catenin Complex in C2S Cells. We have analyzed two proteins of the cadherin–catenin complex, E-cadherin and β-catenin (Boller et al., 1985; Herron and Gordon, 1995), as well as APC, a microtubule-binding protein that also interacts with β-catenin and participates in the transduction processes (Hulsken et al., 1994; Vleminckx, et al., 1997).

Immunohistochemical labeling of E-cadherin was obvious at the periphery of both CB6 and C2S cells (Fig. 4, A and B). E-cadherin was slightly more expressed in Cdx2-overexpressing cells than in controls, as assessed by a modest increase (1.7-fold) in the intensity of the protein band detected by Western blot (Fig. 5), and corroborated at the mRNA level (Fig. 6). In contrast, the mRNAs encoding β-catenin and APC were unchanged in both cell lines, and on the one hand an upregulation of β3 chain mRNA was observed by RT-PCR (Fig. 5) and the corresponding RT-PCR signal (Fig. 6) was more intense in Cdx2-overexpressing cells than in control cells.

Expression of Laminin Constituent Chains and Integrin Subunits Varies in C2S Cells. We analyzed on one hand various constituent chains of laminin isoforms, α1, α2, α3, β1, β3, and γ2, which are associated in laminin-1 (α1/β1/γ1) and laminin-5 (α3/β3/γ3), and on the other hand α6, β1 and β4 integrin subunits (for reviews see Mercurio, 1995 and Simon-Assmann et al., 1995).

As far as laminin chains are concerned, C2S cells displayed on one hand a downregulation of α1 chain mRNA and on the other hand an upregulation of γ2 chain mRNA compared to control cells (Fig. 6). In contrast, the mRNAs encoding the β1 and β3 laminin constituent chains were unchanged in both cell lines and that of the laminin α3 chain was undetectable (not shown).

Concerning integrin subunits, Cdx2-overexpressing cells displayed interesting changes in the α6, β1, and β4 mRNA content and/or in the amount or localization of the corre-
responding proteins. Although the level of $\alpha 6$ mRNA (not shown) and the corresponding protein band revealed on immunoblots of whole cell extracts was similar in C2S and control cells, the intensity of the membrane-associated $\alpha 6$ protein band was significantly increased in C2S cells (Fig. 5). In parallel, immunostaining of the $\alpha 6$ integrin subunit on the ventral side of C2S cells was organized in parallel fibers and was more intense than that found in control CB6 cells, in which only short dots were visible (Fig. 4, compare F to E). These observations suggest that this integrin subunit was recruited in the membrane of C2S cells. An inverse observation was made for the integrin-$\beta 1$ subunit since a rather weak labeling was detected at the base of Cdx2-overexpressing C2S cells, contrasting with the sharp basal staining of small fibers in control CB6 cells (Fig. 4, compare H to G). Consistently, the amount of integrin-$\beta 1$ protein (Fig. 5) and mRNA (Fig. 6) was reduced in C2S cells compared to CB6; Northern blot quantification indicated a 2.5-fold decay of integrin-$\beta 1$ mRNA in these cells (not shown). As far as the integrin-$\beta 4$ subunit is concerned, it was hardly detected in control CB6 cells at both protein (Figs. 4 and 5) and mRNA levels (Fig. 6), as previously reported for parental Caco2 cells (Basson et al., 1992; Fontao et al., 1997). However, immunostained patches were clearly visualized on the ventral side of C2S cells (Fig. 4J), the integrin-$\beta 4$ subunit protein band was clearcut on Western blots of C2S cell membrane extracts (Fig. 5), and the corresponding mRNA was detected in these cells (Fig. 6).

**Some Intracellular Proteins Are Increased in Cdx2-overexpressing Cells.** The expression of intracellular molecules expected to mediate extracellular matrix signals was investigated: HD1, a component of hemidesmosomes that

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**Figure 4.** Immunodetection of E-cadherin (A and B), APC (C and D) integrin-$\alpha 6$ (E and F) integrin-$\beta 1$ (G and H), integrin-$\beta 4$ (I and J), and HD1 protein (K and L) in control CB6 (A, C, E, G, I, and K) and Cdx2-overexpressing C2S cells (B, D, F, H, J, and L) cultured for 5 d. Cells were fixed with 1% paraformaldehyde and permeabilized with 1% Triton X-100 (A–J), or fixed with cold methanol (K and L). Bars, 40 $\mu$m.

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**Figure 5.** Protein expression in control CB6 (lanes 1) and C2S cells (lanes 2) cultured for 5 d and analyzed by immunoblotting experiments. 50 $\mu$g of proteins were loaded in each lane for electrophoresis on 7.5% polyacrylamide gels. $\alpha$-Actinin ($\alpha$-Act), APC, FAK, integrin-$\alpha 6$ (Int-$\alpha 6$), and integrin-$\beta 1$ (Int-$\beta 1$) were analyzed in whole cell extracts. E-cad/m, Int-$\alpha 6$/m, and Int-$\beta 4$/m, respectively, designate E-cadherin, integrin-$\alpha 6$, and integrin-$\beta 4$ present in cell membrane preparations.

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**Figure 6.** RT-PCR analysis of the mRNA expression of E-cadherin, APC, laminin-$\alpha 1$ chain, laminin-$\gamma 2$ chain, integrin-$\beta 1$ subunit, integrin-$\beta 4$ subunit, $\alpha$-actinin, and FAK in control CB6 (lanes 1) and C2S cells (lanes 2) cultured for 5 d. The number of RT-PCR cycles was 30 for E-cadherin, 26 for APC, 26 for $\alpha$-actinin, 28 for laminin-$\alpha 1$, and $\gamma 2$, 26 for integrin-$\beta 1$, 24 for integrin-$\beta 4$, and 28 for FAK. Standard RT-PCR was made with 34B6 primers for 22 cycles.
interacts with α6β4 integrin and cytokeratins (Hieda et al., 1992; Sonnenberg et al., 1993; Fontao et al., 1997), the focal adhesion kinase pp125FAK (Hanks et al., 1992; Schaller et al., 1992), and α-actinin that connects β1-integrin to the actin network (Craig and Pardo, 1979).

We found a slight increase in the expression of α-actinin in C2S cells compared to control CB6 cells at the protein (Fig. 5) and mRNA (Fig. 6) levels. Immunostaining of HD1 protein was almost absent in control CB6 cells (Fig. 4K), as previously shown in parental Caco2 cells (Fontao et al., 1997); however a clear staining was observed on the basal side of the C2S cells (Fig. 4L). Finally, the tyrosine kinase associated with focal adhesions, pp125FAK, and the corresponding mRNA, were present without any obvious changes in C2S and CB6 cells (Figs. 5 and 6).

It is noteworthy that these variations were also recorded, but to a lower extent, in the pooled populations of pCdx2-S– and pCB6–transfected cells. Taken together, these results indicate that Cdx2 overexpression provokes modifications in the level of expression or in the localization of molecules involved in cell–cell interactions, in cytoskeleton organization and transduction processes, and mostly in cell–substratum interactions.

**Adhesion and Aggregation Properties of C2S Cells**

The higher resistance to trypsin treatment displayed by Cdx2-overexpressing cells compared to control cells at confluence, and the modification of expression of molecules involved in cell–cell and cell–substratum interactions prompted us to investigate whether this behavior reflects modifications in the intrinsic adhesion and/or aggregation properties of the cells. For this purpose, C2S and CB6 cell adhesion to plastic or laminin-1 was compared after 1 and 2 h. Laminin-1 coatings increased the attachment of both cell types to the substratum (mean increase of 2.5-fold). However, C2S cells adhered two to three times less than control CB6 cells to plastic as well as to laminin. Adhesion inhibition studies, using antiintegrin antibodies, indicated that antiintegrin-β1 antibody caused an equal 85% inhibition of C2S and CB6 cell attachment to laminin-1. Antiintegrin-β4 and antiintegrin-α6 antibodies failed to inhibit cell attachment of both clones to laminin-1, as already observed in parental Caco2 cells (Orian-Rousseau, V., unpublished results). Aggregation tests indicated that 29% of the starting CB6 cell suspension formed aggregates containing more than four cells after 30 min under slow agitation, whereas the value increased to 49% for Cdx2-overexpressing C2S cells.

The higher capacity of C2S cells to form cell–cell contacts than the control cells could result from the increased expression of E-cadherin in these cells; it may also explain the higher resistance of C2S cells than CB6 cells to trypsin treatment at confluence. On the other hand, the lower capacity of C2S cells to adhere to the substratum could be linked to the decline of integrin-β1 expression, but seems to be independent on the rise of integrin-β4 expression, as confirmed by adhesion inhibition studies with specific antiintegrin antibodies.

**Xenografts of C2S Cells**

C2S cells were injected subcutaneously to nude mice and tumors were recovered after 7 and 12 wk. At 7 wk, expression of the Cdx2 transgene under the control of the cytomegalovirus promoter was retained, as demonstrated by RT-PCR (Fig. 7D). Tumors developed from the C2S cells were twice as large as those derived from control CB6 or parental TC7 cells. Despite the difference in size, both types of tumors displayed glandular structures with a single, polarized, epithelial layer lining a central lumen and delineated by stromal cells derived from the host (Fig. 7A). Controls (not shown) and C2S tumors expressed SI on the apical side of the epithelial cells (Fig. 7B), as well as integrin-β4 subunit at the interface with stromal cells (Fig. 7D). 12 wk after implantation, C2S tumors displayed a five-fold greater weight than controls; they exhibited glands, sporadic villus-like structures (Fig. 7, E and F) and necrotic areas in the center of the tumors, unlike CB6 or TC7 tumors which were almost filled with stromal and granulation tissue. In the villus-like structures, SI (Fig. 7E) and integrin-β4 subunit (Fig. 7F) were detected, respectively, at the apical pole of the polarized epithelial cells and at the basal surface of the cells facing the stroma. The differences observed between C2S and CB6 tumors were also found...
amongst the tumors developed from the pooled populations of transfected cells (not shown).

Cdx2 Expression in TC7 Cells Grown In Vitro on Laminin Coatings

Extracellular matrix components of the basement membrane have been shown to potentiate intestinal cell differentiation (Simon-Assmann et al., 1995). Of particular interest, SI expression is increased in Caco2 cells grown in vitro on laminin coatings (Vachon and Beaulieu, 1995; Basson et al., 1996). Cdx2 overexpression stimulates the expression of several differentiation markers, such as sucrase-isomaltase; thus, we analyzed whether the differentiating effect of laminin could correlate with an increase in Cdx2 expression in Caco2 cells. For this purpose, TC7 cells were grown on standard culture dishes or on dishes previously coated with laminin-1 prepared from EHS tumors. RNA was extracted on day 5 of culture, and analyzed by RT-PCR for Cdx2 expression; SI mRNA was used as a marker of cell differentiation. As shown in Fig. 8 A, laminin coatings promoted cell differentiation, assessed by the higher level of SI mRNA present in cells grown on laminin compared to plastic; noteworthy, the level of Cdx2 mRNA also increased in cells cultured in presence of laminin-1. These results suggest that Cdx2 expression is stimulated by laminin signaling.

Cdx2 Expression in Cellular Clones Deficient in Laminin-α1 Chain

To strengthen the above observations, we took advantage of Caco2 cell clones of the LAMα1-AS series established in our laboratory, in which endogenous expression of the laminin-α1 chain was inhibited by antisense RNA (De Arcangelis et al., 1996). Inhibition of laminin-α1 chain synthesis altered the processes of basement membrane assembly and cell differentiation, as shown by morphological and immunological observations.

We have analyzed the expression of Cdx2 and SI as a function of the residual level of endogenous laminin-α1 mRNA present in three distinct clones of the LAMα1-AS series: LAMα1-AS12, LAMα1-AS20, and LAMα1-AS22, and in one control clone transfected with pCB6. RNA was extracted from cells grown for 5 d. Fig. 8 B shows that SI mRNA expression paralleled the amount of laminin-α1 mRNA present in the cells, confirming that inhibition of laminin-α1 chain by antisense RNA provoked a decay of SI gene expression. Cdx2 expression also well correlated the amount of laminin-α1 mRNA: indeed, clones in which the endogenous production of laminin-α1 RNA was low, displayed a low amount of Cdx2 mRNA, whereas this transcript was higher in clones synthesizing a higher amount of laminin-α1 RNA (Fig. 8 B). To further confirm these findings, an additional experiment was conducted on the LAMα1-AS12 clone, which shows a stringent phenotype of inhibition of laminin-α1 chain production (see De Arcangelis et al., 1996 and Fig. 8 C). This clone, originally grown in culture medium containing 1.2 mg/ml G418, was propagated for several passages in the presence of a low amount of G418 (0.6 mg/ml). After five passages, the endogenous level of laminin-α1 mRNA increased (Fig. 8 C), as already reported (De Arcangelis et al., 1996). Again, there was a parallel between the levels of laminin-α1 chain, SI, and Cdx2 expression, because the upregulation of laminin-α1 mRNA was accompanied by an increase of the level of SI and Cdx2 transcripts (Fig. 8 C).

Comparison of the Phenotypic Modifications Induced by Laminin-1 Coatings and Cdx2 Overexpression

To analyze whether the differentiation-promoting effect induced by laminin-1 can be linked to the phenotypic changes resulting from Cdx2 overexpression, we analyzed the expression of molecules found to be affected by Cdx2 overexpression, in TC7 cells grown on laminin-1 or on plastic, and in the LAMα1-AS12 clone cultured in medium containing a high or a low concentration of G418. This concerned three transcripts that were upregulated in Cdx2-overexpressing cells: the HoxA-9, APC, and integrin-β4 mRNAs; and two transcripts downregulated in these cells: the Cdx1 and integrin-β1 mRNAs.

As shown in Fig. 9, TC7 cells grown on laminin coatings for 5 d displayed an upregulation of HoxA-9, APC, and integrin-β4 mRNA, and a downregulation of Cdx1 and integrin-β1 transcripts, compared to cells cultured on plastic. Similar modifications in mRNA levels were observed in reverted LAMα1-AS12 cells producing endogenous laminin-α1 mRNA (grown in the presence of 0.6 mg/ml G418) compared to cells grown in the presence of 1.2 mg/ml G418. Immunocytochemical observations of integrin-α6 and -β4 subunits in TC7 cells grown on laminin confirm the stimulation of integrin-β4 expression, as highly positive patch-like structures appeared at the ventral side of the cells, and codistributed with α6 positive patches (not shown). In these cells, integrin-β1 subunits were hardly detected.

These data show that laminin-1 (of exogenous or endogenous origin) induces phenotypic changes similar to those observed when Cdx2 homeobox gene is overexpressed, as far as the pattern of gene expression and the recruitment of integrin subunits on the cell base is concerned.
Discussion

Two major conclusions arise from the present study. First, overexpression of a single homeobox gene, Cdx2, causes pleiotropic effects reproducing the properties of differentiated enterocytes lining the villi. These include a rise in differentiation markers such as sucrase-isomaltase and lactase-phlorizin hydrolase, as well as modifications in the expression pattern of molecules involved in cell–cell and mainly in cell–substratum interactions. Second, laminin-1, an extracellular matrix component of the basement membrane, is a potent regulator of the expression of Cdx2. These results suggest that the Cdx2 homeobox gene plays a key role in the cascade of events involved in extracellular matrix–mediated intestinal cell differentiation.

Although it is difficult to extrapolate results obtained from colonic adenocarcinoma cells to the normal intestinal epithelium, several features displayed by Cdx2-overexpressing Caco2 cells can be related to the properties of differentiated enterocytes. For instance, C2S cells show a significant rise of SI and LPH, two markers of the mature enterocytes. They also exhibit a stimulation of β4 integrin subunit, consistent with the fact that, in vivo, this base- membrane receptor chain exhibits an increasing gradient along the crypt–villus axis (Simon-Assmann et al., 1994b). APC, E-cadherin, and the laminin-γ2 chain, preferentially expressed by villus cells (Hermiston and Gordon, 1995; Nathke et al., 1996; Orian-Rousseau et al., 1996), are also upregulated in Cdx2-overexpressing cells. Of interest is the fact that Cdx2 overexpression stimulates the synthesis and/or the recruitment at the cell base of α6 and β4 integrin subunits and of HD1, α6β4 integrin, HD1 together with laminin-5, which comprises the laminin γ2 chain, constitute the major components of type II hemidesmosomes concentrated at the base of villi enterocytes; studies in the future will investigate whether macromolecular hemidesmosomal structures, absent in parental Caco2 cells (Orian-Rousseau et al., 1996; Fontao et al., 1997), are actually assembled in Cdx2-overexpressing cells. The membrane recruitment of integrin-α6 subunits from an intracellular pool of molecules is consistent with observations made for other integrins during keratinocyte differentiation (Lenter and Vetsweber, 1994; Hotchin et al., 1995). Taken together, the present results indicate that Cdx2 impels cells toward the phenotype of villi enterocytes, suggesting that this homeobox gene has a master function in the coordinate process leading to cell differentiation during the continuous renewal of the intestinal epithelium.

The pleiotropic effects of Cdx2 on cell differentiation contrast with the results obtained when Cdx1 expression is modified in Caco2 cells. These differences in vitro may be related to the distinct patterns of expression of both genes in vivo, since CDX1 protein is restricted to undifferentiated crypt cells, whereas CDX2 is located in the nuclei of differentiating enterocytes (James et al., 1994; Subramanian et al., 1995). It is worth noting that the decrease of Cdx1 expression by antisense RNA as well as the overexpression of Cdx2 result in an inhibition of cell proliferation. This, together with the fact that the level of Cdx1 mRNA is reduced in Cdx2-overexpressing cells, may suggest a negative regulatory effect of the CDX2 protein on Cdx1 mRNA expression in enterocytes that pass the crypt–villus junction; positive and negative regulatory elements have been reported in the Cdx1 gene promoter (Hu et al., 1993). A direct interaction between the CDX2 protein and promoter elements of enterocytic markers of differentiation has been demonstrated (Suh et al., 1994; Drummond et al., 1996; Lee et al., 1996; Lambert et al., 1996; Troelsen et al., 1997). However, there is no information concerning the other genes studied here: E-cadherin, APC, α-actinin, the laminin-α1 and -γ2 chains, and the integrin-β1 and -β4 subunits. Recently, several genes belonging to Hox clusters have been proposed to be the targets of Cdx1 during early development of skeletal structures (Subramanian et al., 1995), and we show herein that cells overexpressing Cdx2 display an increase of HoxA-9 mRNA level. It is therefore possible that the phenotype of Cdx2-transfected cells results from the direct interaction of CDX2 protein with the promoters of enterocytic differentiation markers, as well as from indirect effects mediated by secondary regulatory genes such as those belonging to the homeobox gene family.

The results obtained in this study are somewhat unexpected, as regards the possible link between Cdx2 expression and colonic tumorigenesis. Indeed, on the one hand, transfected Caco2 cells producing Cdx2 antisense RNA, as well as transfected intestinal IEC18 cells (personal data), attach poorly to the culture dish, suggesting that some alterations in cell–cell and/or cell–substratum interactions have occurred, like in many cancer cells. This is consistent with the propensity of Cdx2-overexpressing cells to form aggregates, while E-cadherin and APC, two tumor or invasion suppressor molecules, are upregulated (Frixen et al., 1991; Vleminkx et al., 1991; Nathke et al., 1996). We also found in these cells an important decrease in the level of bcl-2 mRNA (our unpublished result), an anti–apoptotic protooncogene deregulated in many cancers (Reed, 1995). These data, together with the fact that there is a dramatic decay of Cdx2 expression in human colonic tumors and in experimentally induced tumors in the rat (Ee et al., 1995; Mallo et al., 1997), suggest a tumor suppressor role of Cdx2 in the gut. Confirmation of this has recently been provided by heterozygous Cdx2 knock-out mice which develop multiple adenomatous polyps and metaplasia in the colon (Chawengsaksophak et al., 1997). However, on the other
hand, Cdx2-overexpressing cells injected in nude mice develop tumors larger than the control ones. Although no clear explanation can be put forward, this unexpected behavior may be related to the higher level of β4-integrin subunit and/or of laminin-γ2 chain in these cells. Indeed, both molecules are possibly linked to tumor formation or invasion (Pyke et al., 1994; Chao et al., 1996). Furthermore, the HT29 cell line expressing high levels of α6/β4 integrin and laminin-5 form significantly larger tumors in nude mice than the poorly expressing parental Caco2 cell line (Simon-Assmann et al., 1994; Orian-Rousseau, V., personal communication).

A major result of this work concerns the relationship between Cdx2 expression and the presence of a basement membrane component: laminin-1. Various investigations have already been carried out, in particular in the liver and mammary gland, to explore the molecular basis of gene regulation by extracellular matrix components (Roskelley et al., 1995). They led to the identification of extracellular matrix–responsive elements in the gene promoters of specific cell differentiation markers (Schmidhauser et al., 1990, 1992; Juliano and Haskell, 1993). Using cell cultures on laminin-1 coatings and a collection of cell clones in which the endogenous production of laminin-α1 chain is inhibited by antisense RNA, we provide strong arguments in favor of a regulatory role played by extracellular matrix components, of endogenous or exogenous origin, on Cdx2 expression. Since additionally, we observed modifications in the expression of some laminin chains and integrin subunits by Cdx2 overexpression, we conclude that homeobox genes and molecules involved in cell–substratum interactions exert reciprocal controls during intestinal cell differentiation. The mechanism whereby laminin-1 regulates Cdx2 in Caco2 cells remains unclear. In particular, we do not know whether Cdx2 is directly controlled by transduction signals resulting from the binding of laminins to their integrin receptors, or whether this effect is indirectly exerted through a control of cell shape (Singhvi et al., 1994; Roskelley et al., 1995), or by promoting the binding of soluble factors to their membrane receptors (Streuli et al., 1995b; Miyamoto et al., 1996). The participation of integrins in mediating the effect of laminin-1 is suggested by the segregation of integrin subunits on the basal side of cells grown on laminin-1 coatings (our unpublished data). In parental Caco2 cells, the primary effect of laminin may involve integrins comprising the β1 chain, because integrin-β4 is virtually absent in these cells. Although the precise downstream signalling pathway is not known, it may require phosphorylation events including pp125FAK or ras-dependent protein kinases, and/or the activation of transcription factors such as STAT or NF-κB (Schlaepfer et al., 1994; Streuli et al., 1995a; Roskelley et al., 1995; Dehdar and Hannigan, 1996; Rosales and Juliano, 1996).

This study indicates that extracellular signals delivered by laminin-1 lead to a modification in the expression of the CDX2 homeoprotein, which in turn provokes changes (a) in the composition of the secreted basement membrane molecules (decay of laminin-α and increase of laminin-γ2 mRNAs) and (b) in the cellular attachment properties and transduction signals (modification of the repertoire of integrins: decay of integrin-β1 and stimulation of integrin-β4 subunits). Since several laminin chains and integrin subunits exhibit a specific distribution along the crypt–villus axis (Beaulieu, 1992; Simon-Assmann et al., 1994b; Perreault et al., 1995; Leivo et al., 1996), we speculate that, in addition to a possible intrinsic program of proliferation/differentiation held by the enterocytes themselves, a molecular code based on the spatial distribution of laminins and integrins may instruct cells to switch from proliferation to differentiation at the crypt–villus junction, during the continuous process of renewal of the intestinal epithelium. Although the complete cascade of events is far from being elucidated, the present results provide new insight to approach the molecular mechanisms of intestinal cell differentiation in relation with epithelial–mesenchymal cell interactions, extracellular matrix components and the control of homeobox gene expression. This study also opens the question whether the Cdx2 decline observed in colon cancers results from regulatory changes associated with some alterations of extracellular matrix signaling, and/or with modifications of the transduction processes linked to oncogenic signaling.

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