Stimulation of Cdx1 by oncogenic β-catenin/Tcf4 in colon cancer cells; opposite effect of the CDX2 homeoprotein

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Abstract The homeobox gene Cdx1 is a regulator of intestinal epithelial cell proliferation and differentiation. Using a transfection approach, we showed here that the oncogenic activation of the β-catenin pathway stimulates the endogenous expression of the Cdx1 mRNA as well as the activity of the Cdx1 promoter in cancer cells of the human colon. Reciprocally, the paralogue homeobox gene Cdx2 exerts an inhibitory effect on the basal and on the β-catenin-stimulated activity of the Cdx1 promoter. The inhibitory effect of CDX2 requires the intact homeodomain. It is not dependent on canonical CDX binding sites in the Cdx1 promoter nor on the cis-elements specifically targeted by the β-catenin/Tcf complex. We conclude that the oncogenically activated β-catenin and CDX2 have opposite and independent effects on the Cdx1 homeobox gene.

Key words: Homeobox gene; β-Catenin; Colon cancer; Human

1. Introduction

The mammalian homologues of the Drosophila caudal homeobox gene, Cdx1 and Cdx2, are expressed in many organs during gastrulation, and they become restricted to the intestinal epithelium during foetal and adult life (for a review, see [1]). In the gut, Cdx1 is expressed in the crypts where epithelial cells are proliferating, whereas Cdx2 is mainly expressed in the villi lined by differentiated cells [2]. Consistent with their distribution along the crypt–villus axis, Cdx1 stimulates cell proliferation while Cdx2 reduces proliferation, and both genes promote cell differentiation [3–6]. Thus, these homeobox genes participate in the control of intestinal homeostasis by regulating the equilibrium between proliferation and differentiation during the constant renewal of the gut epithelium.

Alteration of cell proliferation/differentiation is thought to be a major event in the initiation and progression of cancer. Colorectal cancer is a multistep process linked to the inactivation or loss of expression of tumour suppressor genes and to the constitutive activation or overexpression of proto-oncogenes. Activation of the β-catenin/Tcf pathway, through the loss of function of the APC tumour suppressor that antagonises β-catenin signalling or through the oncogenic activation of β-catenin itself, represents an early and frequent event in colorectal cancers [7,8]. Besides, several studies have reported that colon tumourigenesis also shows modification in the expression of Cdx1 and Cdx2. Indeed, Cdx2 decreases in relation with the tumour grade [9], and a decline of Cdx1 also occurs in the majority of colon carcinomas [10–12]. However, we found that Cdx1 is upregulated in more than one third of the polyps, an early stage during tumour progression (unpublished results). Moreover, ectopic expression of Cdx1 occurs in adenomas that develop in digestive organs outside the gut, like the oesophagus, the stomach and the liver [12,13]. In addition, Cdx1 has a cell transformation capacity assessed by anchorage-independent growth and tumour development of Cdx1-expressing cells [14] and it is stimulated by oncogenic ras [15]. Together, these findings suggest that Cdx1 has a pro-oncogenic potential. On the contrary, the decline of Cdx2 in colorectal cancers [9], along with the fact that this gene inhibits cell growth [3–5] and is downregulated by oncogenic ras [15], led to attribute a tumour suppressor function to Cdx2.

Little is known about the molecular mechanism(s) that regulate Cdx1. We have previously reported that this homeobox gene is a target of Wnt/β-catenin/Tcf signalling in embryonic stem cells as well as during foetal endoderm development [16]. However, it is not known whether the oncogenic activation of this pathway in colorectal cancer cells also stimulates Cdx1. Reciprocally, forced expression of CDX2 has been shown to reduce the level of Cdx1 mRNA in colon cancer cells, raising the possibility that Cdx2 exerts an inhibitory effect on Cdx1 [4]. The aim of this study was to investigate whether Cdx1 is targeted by the oncogenic activation of the β-catenin pathway in colon cancer cells, and whether there is a relation between β-catenin and CDX2.

2. Materials and methods

2.1. Cell culture and transfection

The human colon adenocarcinoma cell lines Caco2TC7 and HT29, and the embryonic kidney cells HEK293 were cultured in DMEM with 20% (Caco2TC7) and 10% (HT29, HEK293) foetal calf serum. The fibroblastic cell line RC9 was raised in our laboratory from the intestinal mesenchyme of neonatal rats and maintained in culture in DMEM with 10% foetal calf serum. For transfections, cells were incubated 3 h with 3 μl Exgen500 (Euromedex) per 1 μg DNA, and the transfection medium was then replaced with fresh culture medium. Transfected cells were subsequently incubated at 37°C for 24 h before analysis.

2.2. Plasmids

The reporter luciferase plasmids pΔPS-Luc and pΔAN-Luc containing 1.5 and 0.7 kb of the murine Cdx1 promoter, the plasmid pΔPS-TBE34m-Luc in which the two proximal Tcf-binding elements (TBEs) of the Cdx1 promoter were mutated, and the expression plasmid

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pCdx2S encoding the murine CDX2 homeoprotein have been described [4,15,16]. Variant forms were derived from pCdx2S using site-directed mutagenesis (Promega, details are available upon request): pHA-Cdx2 encodes an HA-tagged version of CDX2; in pHA-Cdx2-232, the codon encoding Trp232 was changed into a stop codon; pHA-Cdx2-Δ1 has an internal 33-bp deletion resulting in a form of HA-CDX2 lacking Ala217 to Val229. For this study, we also used pS33A-βcatenin encoding the oncogenic form of β-catenin [17], and pcDNA3-TCF4E-myc and pcDNA3-ΔNTCF4E-myc encoding, respectively, myc-tagged Tcf4 and the N-truncated dominant negative form of Tcf4 [18]. The reporter plasmid pSI-Luc containing the fragment −370/+30 of the human sucrase–isomaltase promoter inserted in pGl3-Basic (Promega) has been kindly provided by Dr M. Rousset (Inserm U.405, Paris, France). The plasmid pMACS-K^k was from the MACSelect kit (Miltenyi Biotec). pRL-TK (Promega) was used to standardise cell transfections, and pBluescript (Stratagene) was used to correct the amount of DNA during transfections.

2.3. RNA analysis

Caco2TC7, HT29 or RC9 cells (2×10^6) were transfected with 5 μg pS33A-βcatenin, 3 μg pcDNA3-TCF4E-myc and 2 μg pMACS-K^k. Controls were transfected with 8 μg pBluescript and 2 μg pMACS-K^k. After 24 h, cells were trypsinised, labelled with microbeads coated with anti-$H2K^k$ antibody, and loaded on a column for magnetic separation according to the protocol described in the MACSelect kit (Miltenyi Biotec). RNA was extracted from the immunoselected cells using Tri-Reagent (Euromedex) and analysed by semi-quantitative RT-PCR under standard conditions previously established to monitor the Cdx1 and GAPDH transcripts [4,15]. The PCR products were electrophoresed on 3% agarose gels and analysed using an Imaging Densitometer (GelDoc 1000, Bio-Rad). The identity of the PCR products was confirmed by direct sequencing.

2.4. Luciferase measurement

Caco2TC7, HT29 or HEK293 cells (1.5×10^5) were transfected with 1 μg of luciferase reporter plasmid and the appropriate expression plasmid(s) at 0.5 μg, unless otherwise stated. They were cotransfected with 0.02 μg pRL-TK (Promega) for normalisation. After 24 h, cells were harvested for luciferase measurements using the Dual Luciferase Assay (Promega). At least three independent experiments were performed in triplicate for each condition of transfection.

2.5. Western blotting

HEK293 cells (2×10^6) were transfected with 5 μg pBluescript or pHA-Cdx2. After 24 h, cells were washed in PBS, centrifuged at $2500\times g$ and resuspended in two pellet volumes of lysis buffer composed of 200 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% glycerol, 0.5 mM DTT and protease inhibitors (Sigma). NaCl was added to a final concentration of 420 mM, and cells were lysed by two rounds of thaw/freeze at −80°C. Cell extracts were centrifuged at $12\,000\times g$ and the supernatant was stored at −80°C [19]. The presence of CDX2 in the extracts (6 μg) was analysed by standard SDS–PAGE (12% polyacrylamide) and Western blotting using anti-HA monoclonal antibody (Eurogentec, dilution 1:1000). The mouse monoclonal was revealed using peroxidase-coupled anti-mouse antibody (Amersham, dilution 1:4000) and the ECL detection kit (Amersham).

2.6. Immunocytology

HT29 cells (4×10^4) were transfected with 0.25 μg of pHA-Cdx2, pHA-Cdx2-232 or pHA-Cdx2-Δ1. After 24 h, cells were fixed for 20 min in 4% paraformaldehyde. They were incubated in PBS containing 0.25% Triton X100 and anti-HA antibody (Santa Cruz Biotechnology, dilution 1:200) and then with anti-rabbit antibody coupled to Alexa-568 (Molecular Probes, dilution 1:1200) in PBS. Nuclei were stained with Hoechst-33258 (10 μg/ml, Sigma). Fluorescent staining was visualised using an Axiophot microscope (Zeiss).

3. Results and discussion

We have reported that the Cdx1 homeobox gene is a target of the Wnt/β-catenin/Tcf pathway during the normal process of development in mammals [16]. Since the alteration of this pathway is a very frequent and early event during colorectal tumourigenesis, we have investigated if Cdx1 is also targeted

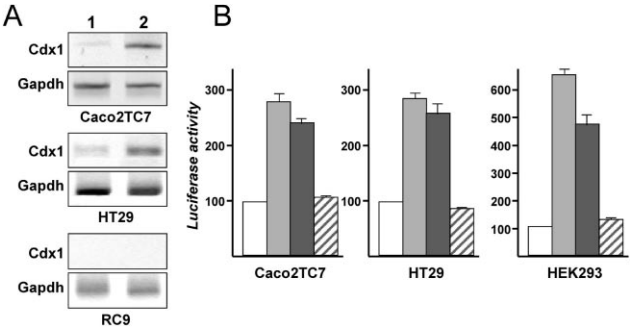

Fig. 1. Stimulation of Cdx1 expression by oncogenic β-catenin in colon cancer cells. A: Human colon cancer cells Caco2TC7 and HT29, or mesenchymal intestinal cells RC9 were transfected with pBluescript (lane 1) or with pS33A-βcatenin and pcDNA3-TCF4E-myc (lane 2), together with pMACS-K^k. RNA was extracted from cells immunoselected for the presence of $H2K^k$ at the surface, and analysed by RT-PCR for Cdx1 and Gapdh mRNA expression. B: Caco2TC7 and HT29 cells or human embryonic kidney cells HEK 293 were transfected with 1 μg of the reporter plasmids pΔPS-Luc (grey), pΔAN-Luc (black) or pΔPS-TBE34m-Luc (hatched), and with 0.5 μg of pS33A-βcatenin and 0.25 μg of pcDNA3-TCF4E-myc. The plasmids pS33A-βcatenin and pcDNA3-TCF4E-myc were replaced by pBluescript in the controls (open). Cells were cotransfected with 0.02 μg pRL-TK for normalisation. Luciferase activity driven by the promoter was related to renilla luciferase and expressed as arbitrary units; the value of 100 was attributed to the controls.

by the oncogenic activation of this pathway in human colon cancer cells. To this end, two human colon adenocarcinoma cell lines, Caco2TC7 and HT29, were transfected with the plasmids pS33A-βcatenin and pcDNA3-TCF4E-myc that encode the oncogenic form of β-catenin and Tcf4, while controls were transfected with pBluescript. Cells were simultaneously cotransfected with a limited amount of pMACS-K^k, which encodes the membrane-anchored $H2K^k$, to select the successfully transfected cells, as they expressed $H2K^k$ at their surface. As shown in Fig. 1A, the level of Cdx1 mRNA was low in control Caco2TC7 and HT29 cells, and it increased in both cell lines transfected with the plasmids encoding the oncogenic form of β-catenin and Tcf4. Unlike the epithelial cells, no Cdx1 mRNA was detected in the intestinal mesenchymal cells RC9, either control or transfected to overexpress the oncogenic β-catenin and Tcf4. Caco2TC7 or HT29 cells were then transfected with pS33A-βcatenin and pcDNA3-TCF4E-myc, and with the luciferase reporter plasmid pΔPS-Luc or pΔAN-Luc containing, respectively, 1.5 or 0.7 kb of the Cdx1 promoter (Fig. 1B). The activity of both reporters was stimulated by 2.5-fold as compared to controls (Fig. 1B). This stimulatory effect was abolished by replacing pcDNA3-TCF4E-myc with pcDNA3-ΔNTCF4E-myc encoding a dominant negative form of Tcf4 unable to interact with β-catenin (not shown). It was also prevented when the two TBEs of the Cdx1 promoter, previously identified as the targets of β-catenin signalling during development [16], were destroyed by point mutagenesis in pΔPS-TBE34m-Luc (Fig. 1B). Finally, when intestinal mesenchymal RC9 cells, instead of the epithelial cell lines, were transfected with pΔPS-Luc, luciferase activity was not above the background, and it was not stimulated by cotransfection with pS33A-βcatenin and pcDNA3-TCF4E-myc (not shown). Taken together, these data demonstrate that the oncogenic activation of β-catenin stimulates the endogenous expression of the Cdx1 mRNA and the transcrip-

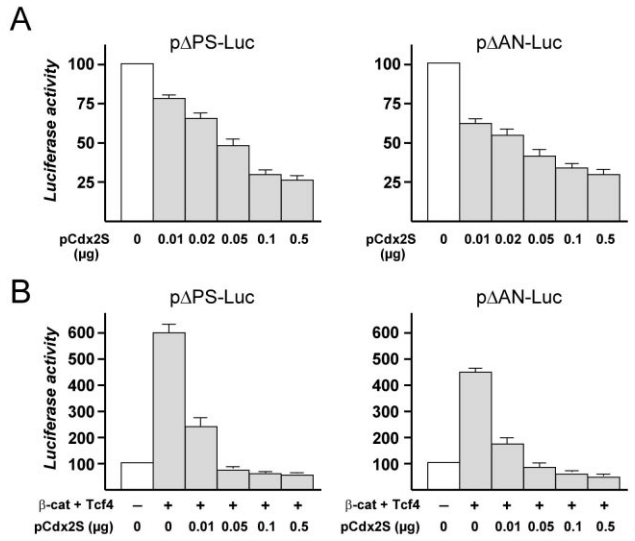

Fig. 2. CDX2 inhibits the basal and β-catenin-stimulated activity of the Cdx1 promoter. A: HEK293 cells were transfected with 1 μg of the reporter plasmid pΔPS-Luc or pΔAN-Luc, without or with increasing amounts of pCdx2S to express the CDX2 protein, and with pRL-TK to normalise transfections. B: Same as in A except that cells were also cotransfected with 0.5 μg of pS33A-βcatenin and 0.25 μg of pcDNA3-TCF4E-myc (β-cat + Tcf4) to stimulate the activity of the Cdx1 promoter. Luciferase activity driven by the promoter was related to renilla luciferase and expressed as arbitrary units; the value of 100 was attributed to the controls.

tional activity of the Cdx1 promoter in colon cancer cells, and that this effect is cell-type specific. The fold stimulation of the Cdx1 promoter activity was lower in Caco2TC7 and HT29 cells than in the widely used HEK293 cells (Fig. 1B, see also [16]), most likely because β-catenin signalling is constitutively activated in both colon cancer cell lines, whereas it is not in the embryonic kidney cells HEK293.

During the course of these experiments, we have also analysed the expression of the Cdx1 paralogue in the intestine, Cdx2, and we failed to demonstrate any obvious change by oncogenic β-catenin/Tcf4 (not shown). Since Cdx1 and Cdx2 play opposite and complementary roles in the regulation of intestinal epithelial cell behaviour [3–6], we have investigated whether the CDX2 homeoprotein has an effect on the basal and/or on the β-catenin-stimulated activity of the Cdx1 promoter. These experiments were performed using HEK293 cells because the stimulation of Cdx1 by oncogenic β-catenin is higher in these cells and because they do not express CDX2 endogenously. Cells were cotransfected with the Cdx1 reporter plasmid pΔPS-Luc or pΔAN-Luc and with pCdx2S that encodes the murine CDX2 protein. As shown in Fig. 2A, the transcriptional activity of both Cdx1 promoter segments was decreased in the presence of CDX2 protein, corroborating our previous finding that the level of Cdx1 mRNA is reduced in colon Caco2TC7 cells stably transfected to overexpress CDX2 [4]. The effect of CDX2 was dose dependent, and a significant inhibition already occurred at a dose as low as 0.01 μg pCdx2S. This prompted us to investigate whether CDX2 can antagonise the stimulatory effect exerted by oncogenic β-catenin on the Cdx1 promoter. For this purpose, HEK293 cells were cotransfected with pΔPS-Luc or pΔAN-Luc, with pS33A-βcatenin and pcDNA3-TCF4E-myc, and with pCdx2S at different doses. Fig. 2B indicates that the four to six fold stimulation of the Cdx1 promoter activity by oncogenic β-catenin/Tcf4 was abrogated by the addition of increasing amounts of pCdx2S. As for the basal activity of the Cdx1 promoter, the β-catenin-stimulated activity was also inhibited at the lowest concentration of pCdx2S used for this study (0.01 μg) (Fig. 2A,B). These results indicate that the CDX2 homeoprotein is a potent inhibitor of the basal and of the β-catenin-stimulated activity of the Cdx1 promoter.

In an attempt to identify the mechanism of action of the CDX2 protein, we asked whether the inhibitory role of CDX2 on Cdx1 expression was dependent on Tcf4 bound to the TBEs of the Cdx1 promoter. Indeed, it has already been shown that DNA-binding factors of the Tcf family act as either transcription activators or inhibitors depending on the cofactors with which they interact (β-catenin or TLE1, respectively). To address this question, we have compared the effect of CDX2 on the original pΔPS-Luc reporter plasmid and on pΔPS-TBE34m-Luc, in which the two TBEs of the Cdx1 promoter were mutated. We showed that pCdx2S exerted the same inhibitory effect on pΔPS-TBE34m-Luc as on pΔPS-Luc either in the absence or in the presence of the plasmids coding for oncogenic β-catenin and Tcf4 (Fig. 3). This indicated that the inhibition of the Cdx1 promoter by CDX2 is not mediated by the TBEs needed for the stimulation by β-catenin/Tcf4. Hence CDX2 and β-catenin exert opposite effects on Cdx1 using distinct mechanisms.

We next investigated whether an intact CDX2 homeodomain was required for its inhibitory effect. For this purpose, we have constructed the plasmid pHA-Cdx2 that coded for the HA-tagged version of CDX2, and two derived plasmids on the basis of mutations reported in homeoproteins other than CDX2. In pHA-Cdx2-232, the codon corresponding to

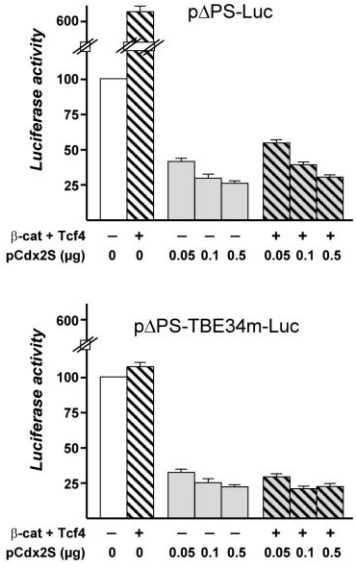

Fig. 3. Mutation of the TBEs of the Cdx1 promoter does not prevent inhibition by CDX2. HEK293 cells were transfected with 1 μg of the reporter plasmid pΔPS-Luc or pΔPS-TBE34m-Luc in which the two TBEs of the Cdx1 promoter were disrupted, and in the presence or in the absence of pS33A-βcatenin and pcDNA3-TCF4E-myc. The plasmid pCdx2S was added as indicated, and cells were cotransfected with pRL-TK for normalisation. Luciferase activity driven by the promoter was related to renilla luciferase and expressed as arbitrary units; the value of 100 was attributed to the controls.

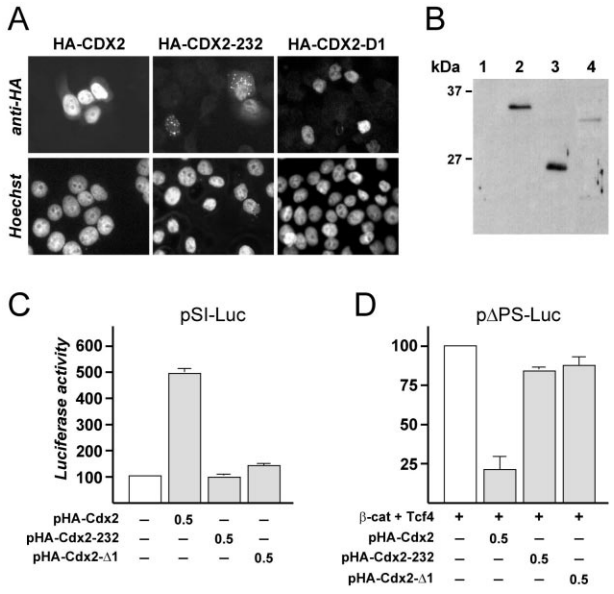

Fig. 4. Disruption of the CDX2 homeodomain prevents the inhibitory function of the protein. A: HT29 cells were transfected with the plasmid pHA-Cdx2, pHA-Cdx2-232 or pHA-Cdx2-Δ1, and the corresponding protein was revealed using anti-HA antibody (upper panel). The cell nuclei were labelled with Hoechst (lower panel). B: Western blotting using anti-HA antibody of control HT29 cells (lane 1) and of cells transfected with pHA-Cdx2 (lane 2), pHA-Cdx2-232 (lane 3) or pHA-Cdx2-Δ1 (lane 4). C: The activity of the CDX2 variants was analysed by transfection of HT29 cells with 0.5 μg of the appropriate expression plasmids and with 1 μg of the reporter target pSI-Luc that contains a fragment of the sucrase–isomaltase promoter. Cells were cotransfected with pRL-TK for normalisation. C: Same as in B except that HEK 293 cells were transfected with pΔPS-Luc instead of pSI-Luc. Cells were also cotransfected with 0.5 μg of pS33A-βcatenin and pcDNA3-TCF4E-myc (β-cat + Tcf4) to stimulate the activity of the Cdx1 promoter. Luciferase activity driven by the promoter was related to renilla luciferase and expressed as arbitrary units; the value of 100 was attributed to the controls.

Trp232 within helix-3 of the homeodomain was changed into a stop codon to delete the 79 amino acids of the C end of CDX2. The corresponding mutation in the human HoxA13 gene is responsible for the Hand–Foot–Genital syndrome [20]. The second plasmid, pHA-Cdx2-Δ1, encodes a protein variant in which 11 amino acids were deleted at the boundary between helix-2 and helix-3 of the homeodomain (from Ala217 to Val229). Deletion of the corresponding Δ1 sequence in the engrailed homeoprotein prevents nuclear export and intercellular transfer [21,22]. Immunofluorescence staining with anti-HA antibody in transfected HT29 cells confirmed the nuclear localisation of HA-CDX2 although a faint staining was also detected in the cytoplasm; the HA-CDX2-232 protein was nuclear and also showed a punctuate staining in the cytoplasm, while HA-CDX2-Δ1 exhibited a weak signal only in the nucleus (Fig. 4A). Western blotting of the transfected cells confirmed that proteins of expected size were produced in the cells, although HA-CDX2-Δ1 was present at a relatively low level (Fig. 4B). The activity of the variant forms of CDX2 was checked by cotransfection with the sucrase–isomaltase promoter as reporter target (Fig. 4C). HA-CDX2 stimulated this promoter as did the wild-type protein, but HA-CDX2-232 and HA-CDX2-Δ1 were both inactive. When the plasmids pHA-Cdx2-232 or pHA-Cdx2-Δ1 were introduced into HEK293 cells together with pΔPS-Luc, pS33A-βcatenin and pcDNA3-TCF4E-myc, the Cdx1 promoter was not inhibited, indicating that an intact homeodomain is required for the inhibitory effect of CDX2 (Fig. 4D).

The Cdx1 promoter fragment inserted in pΔPS-Luc contains two putative binding sites for CDX proteins, T/CATA-AAT/G [23]. The upstream-most site does not mediate the inhibitory effect of CDX2 since it is not present in the fragment contained in pΔAN, which is inhibited by CDX2 (see Fig. 2). The second site corresponds to the TATA-box of the Cdx1 gene. An inhibitory role of CDX2, associated to TATA-box binding, has already been reported in the case of the calbindin-D9K gene [24]. To investigate whether CDX2 can bind the Cdx1 TATA-box, EMSA was performed using nuclear extracts of pHA-Cdx2-transfected cells and a labelled double-stranded oligonucleotide overlapping the Cdx1 TATA-box. No specific band supershifted with anti-CDX2 antibody was detected in these experiments. We also failed to detect any bandshift using in vitro transcribed/translated CDX2 (not shown).

In summary, we have demonstrated that the oncogenic activation of the β-catenin/Tcf pathway stimulates Cdx1 expression in adult colorectal cancer cells. This is in line with the stimulation of Cdx1 by Wnt during foetal development [16]. In addition we showed that the CDX2 homeoprotein is a potent inhibitor of the basal and β-catenin-stimulated activity of the Cdx1 promoter. Although the mechanism by which CDX2 downregulates Cdx1 has not been elucidated, one can conclude that the opposite effects of CDX2 and β-catenin are independent. Although we were not able to demonstrate any interaction between CDX2 and the Cdx1 TATA-box in vitro, we cannot rule out that this occurs in vivo or that CDX2 interacts with unconventional binding sites to be identified in the Cdx1 promoter. Alternatively, the effect of CDX2 could be indirect. Indeed, a number of homeoproteins have been shown to exert a transcriptional repression effect in a DNA-binding-independent fashion in Drosophila [25–27]. Moreover, the activity of p300/CBP acetyltransferases, which bind to and potentiate the function of β-catenin [28,29], can be inhibited by Hox proteins via a non-DNA-dependent mechanism involving protein–protein interactions through the homeodomain helix-3 [30]. The opposite effects of β-catenin and CDX2 on Cdx1 gene expression correlate well with the oncogenic role of β-catenin signalling and with the tumour suppressor function attributed to CDX2. We propose that the balance between activated β-catenin and CDX2 may participate in the control of Cdx1 expression, which in turn contributes to the regulation of cell growth and resistance to apoptosis [6,14].

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