A functional role of Cdx2 in β-catenin signaling during transdifferentiation in endometrial carcinomas

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Nuclear β-catenin is required for changes in morphology from glandular to morular phenotypes of endometrial carcinoma (Em Ca), cells, with activation of p14ARF/p53/p21Waf1 and alteration of p16INK4A/pRb pathways. Having demonstrated previously that the homeodomain transcription factor Cdx2 increases markedly during intestinal epithelial cell differentiation, we have examined its effects in β-catenin signaling during transdifferentiation of Em Ca cells. In clinical cases, Cdx2 immunoreactivity, along with increased mRNA signals, was found to overlap with nuclear accumulation of β-catenin and p21Waf1 in morules, demonstrating an inverse correlation with cell proliferation. In cell lines, overexpression of active form β-catenin resulted in a significant increase in endogenous Cdx2 expression at both mRNA and protein levels. Furthermore, the Cdx2 promoter was activated by T-cell factor 4 (TCF4)-independent activated β-catenin, as well as Cdx2 itself, through the region from −39 to +9 bp relative to transcription start site. Cells over-expressing exogenous Cdx2 showed high levels of p21Waf1 expression due to stabilization of the mRNA status, resulting in significant decrease in the proliferation rate, in contrast to the lack of apparent changes in morphology. Moreover, transfected Cdx2 could inhibit β-catenin/TCF4-mediated transcriptional activation of target genes, including p14ARF and cyclin D1, probably through indirect mechanisms. These data suggest that over-expression of Cdx2 mediated by nuclear β-catenin and Cdx2 itself can cause an inhibition of Em Ca cell proliferation through up-regulation of p21Waf1 expression, modulating β-catenin/TCF4-mediated transcription. We therefore conclude that an association between Cdx2 and β-catenin signaling may participate in induction of transdifferentiation of Em Ca cells.

Introduction

The mammalian homologs of Drosophila Caudal homeobox genes, Cdx1 and Cdx2, encode nuclear transcription factors, which are considered to be involved in establishment and maintenance of cell differentiation during development (1–3). However, their roles in regulation of cell proliferation appear complex. For example, Cdx1 expression can promote cell proliferation in the intestinal crypts and Cdx2 can increase division by inducing growth factor gene expression in IEC-6 cells (4–7). In contrast, Cdx1 can regulate intestinal epithelial cell proliferation by inhibiting progression through G1/S via modulation of cyclin D1 and D2 protein levels (8). In addition, Cdx2 expression can lead to activation of the intestine-specific genes and inhibition of cell proliferation, resulting in an induction of intestinal epithelial cell differentiation (9,10).

Members of the Wnt-signaling pathway also have diverse and important functions during development in a wide range of multicellular organisms (11,12). The cytoplasmic β-catenin, a key player in the Wnt-signaling cascade, is tightly regulated by ubiquitin–proteasome pathways, but the increased levels due to escape from glycogen synthase kinase-3β catalysis cause interactions with high mobility group -box transcriptional factors [lymphoid-enhancer factor LEF (TCF) in nuclei, resulting in gene expression that mediates the downstream effects of Wnt (13–15)].

Focal squamous differentiation into the morular phenotype is a common event in endometrial carcinomas (Em Cas), closely linked with an increased probability of survival (16–18). Our previous studies showed that nuclear accumulation of β-catenin is a critical factor in triggering such transdifferentiation toward the morular phenotype of Em Ca cells (19–21). In endometrioid-type ovarian carcinomas, nuclear β-catenin is also considered to be an independent good prognostic factor, involving the genesis of so-called ‘adenoacanthomas’ (tumor with morules) (22). The available findings lead us to speculate that elucidation of the mechanisms may provide a clue to understanding the biological significance of such transdifferentiation events and the central role of β-catenin in carcinogenesis.

Observations from a number of laboratories suggest that effects of Cdx2 on β-catenin signaling contribute to regulation of cell growth and differentiation in intestinal and colonic tissues (23,24). However, it has remained unclear whether such an association exists during transdifferentiation toward the morular phenotype of Em Ca cells. To examine this question, we here determined the expression of Cdx2, along with β-catenin, Cip1/Kip1 and Ink4A Cdk inhibitors, and G1 cyclins, using clinical samples of Em Ca, as well as cell lines.

Materials and methods

Clinical cases

A total of 47 of grade 1 or 2 endometrioid-type Em Cas with morules, surgically resected at Kitasato University Hospital during 2001–2005, were selected, according to the criteria of the World Health Organization classification (1994). Of these, 19 cases overlapped with those used in our previous studies (16–18). In addition, 30 biopsy samples of normal endometrial tissues, including 10 in the proliferative and 20 in the secretory phase (10 early and 10 late stages), were also investigated. All tissues were routinely fixed in 10% formalin and embedded in paraffin wax.

Antibodies

Antibodies to β-catenin (clone 14; Transduction Laboratory, Lexington, KY), Cdx2 (Cdx2-88; BioGenex, San Ramon, CA), p21Waf1 (EA10; Oncogene Res. Prod., Boston, MA), p27Kip1 (clone 57; Transduction Laboratory), total pRb (G3-245; BD Biosciences Pharmingen, San Jose, CA), phospho-pRb (Ser807/811) (ppRb) (Cell Signaling Technology, Beverly, MA), cyclin A (66E; Novocastra Laboratories Ltd, Newcastle, UK), cyclin DI (DCS-6; Dako, Copenhagen, Denmark), p16INK4A (sc-468, Santa Cruz Biotechnology, Santa Cruz, CA), Ki-67 (Dako), HA (Y-11, Santa Cruz Biotechnology) and β-actin (Sigma Chemical Co, St Louis, MO, USA) were employed in this study.

Immunohistochemistry

Immunohistochemistry was performed using new sets of serial sections of Em Ca tissues, as described previously (19–21). For evaluation of immunostaining, areas within tumors were subdivided into morules and surrounding glandular carcinoma (Sur-Ca) lesions, as described previously (16–18). Briefly, nuclei immunopositive for β-catenin, Cdx2, p21Waf1 and Ki-67 were counted for at least 1000 tumor cells in five randomly selected fields of Sur-Ca lesions and at least 700 cells in all morular areas within tumors in each case. Labeling indices (LI) were then calculated as numbers per 100 cells. Scoring for p27Kip1 and total pRb immunoreactivity was also performed according to the method described previously (19–21).

Abbreviations: ChIP, chromatin immunoprecipitation; Em Ca, endometrial carcinoma; LI, labeling index; PCR, polymerase chain reaction; Sur-Ca, surrounding glandular carcinoma; TCF4, T-cell factor 4.
Briefly, the percentage of immunopositive cells in the total tumor cell population was subdivided into five categories as follows: 0, all negative; 1, <30% positive cells; 2, 30–50%; 3, 50–70% and 4, >70%. The immunointensity was also subclassified into four groups as follows: 0, negative; 1+, weak; 2+, moderate and 3+, strong. Immunoreactivity scores for both morular and Sur-Ca lesions for each case were produced by multiplication of the two values.

In situ hybridization for Cdx2 mRNA
The Cdx2 cDNA sequence (containing nucleotides 873–1426; GenBank accession number NM001265) was subcloned into the pCR II vector (Invitrogen, Carlsbad, CA) by a polymerase chain reaction (PCR)-based strategy, using specific primers (supplementary Table S1 is available at Carcinogenesis Online), and riboprobes were generated by in vitro transcription using T7 or Sp6 RNA polymerases in the presence of digoxigenin-labeled UTP. In situ hybridization assays were carried out using the GenPoint Tyramide Signal Amplification System (Dako), according to the manufacturer’s instructions.

Plasmids
A full-length cDNA for HA-tagged Cdx2 (NM001265) was generated by PCR and subcloned into pcDNA3.1 (Invitrogen). The identity was confirmed by sequencing prior to use. Expression plasmids for active form and subcloned into pcDNA3.1 (Invitrogen). The identity was confirmed by sequencing prior to use. The sequences of PCR primers employed in our study are listed in supplementary Table S1 (available at Carcinogenesis Online).

Transient transfection and luciferase reporter assays
Transfection was carried out using LipofectAMINE PLUS (Invitrogen), in duplicate or triplicate, as described previously (19–21). The pRL-TK plasmid (Promega) was used to normalize for transfection efficiency, and luciferase activity was assayed with the Dual-luciferase reporter assay system (Promega).

Reverse transcription–PCR
cDNAs were synthesized from 2 μg of total RNA and amplification of Cdx2 and p21Waf1 mRNAs was carried out in the exponential phase (20–28 cycles) to allow comparison under identical reactions, using specific primers (supplementary
Table S1 is available at *Carcinogenesis* Online. The GAPDH gene was also amplified as an internal control, as described previously (21).

**Western blot assays**

Total cellular proteins were isolated using RIPA buffer [50 mM Tris–HCl (pH 7.2), 1% NP-40, 0.5% sodium deoxycholate and 0.1% sodium dodecyl sulfate] and aliquots of 1–10 μg proteins were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to membranes and probed with primary antibodies, coupled with the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Tokyo, Japan).

**Chromatin immunoprecipitation assays**

Chromatin immunoprecipitation (ChIP) analysis was performed using a ChIP assay kit (Upstate Biotechnology, Lake Placid, NY, USA), as described by D’Amico et al. (27). Briefly, after transient cotransfection of the pGL3B–[−760/+261]Cdx2 reporter construct with either pcDNA3.1-HA-Cdx2 or the empty vector, cells were cross-linked with formaldehyde. Cell lysates were sonicated to shear DNA to lengths between 200 and 1000 bp and diluted 10-fold in ChIP dilution buffer. Chromatin solutions were then precipitated overnight using 4 μg of anti-Cdx2 antibody or rabbit IgG as negative control. After proteinase K digestion, DNAs were extracted and were analyzed by PCR. ChIP analysis was conducted with a reduction in the number of cycles from 30 to 25, using three specific primers.

**Immunofluorescence**

After cotransfection of pcDNA3.1-β-catenin and pcDNA3.1-HA-Cdx2 into Ishikawa cells, they were incubated with primary antibodies to β-catenin and Cdx2.

**Table I.** Correlations among markers investigated in endometrial carcinomas with morules

<table>
<thead>
<tr>
<th>Markers</th>
<th>Nuc-β-catenin LI r (P)</th>
<th>Cdx2 LI r (P)</th>
<th>p21^{WAF1} LI r (P)</th>
<th>p27^{kip1} IHC score r (P)</th>
<th>Total pRb IHC score r (P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cdx2 LI</td>
<td>0.84 (&lt;0.0001)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>p21^{WAF1} LI</td>
<td>0.59 (&lt;0.0001)</td>
<td>0.63 (&lt;0.0001)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p27^{kip1} IHC score</td>
<td>−0.23 (0.037)</td>
<td>−0.26 (0.03)</td>
<td>0.10 (0.38)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total pRb IHC score</td>
<td>−0.53 (&lt;0.0001)</td>
<td>−0.51 (0.0005)</td>
<td>−0.31 (0.017)</td>
<td>0.19 (0.16)</td>
<td></td>
</tr>
<tr>
<td>Ki-67 LI</td>
<td>−0.77 (&lt;0.0001)</td>
<td>−0.73 (&lt;0.0001)</td>
<td>−0.46 (&lt;0.0001)</td>
<td>0.26 (0.03)</td>
<td>0.77 (&lt;0.0001)</td>
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r, Spearman’s correlation coefficient and IHC, immunohistochemistry.

**Fig. 2.** Transactivation of Cdx2 by β-catenin and Cdx2. (A) Changes in protein and mRNA levels of endogenous Cdx2 were examined in Ishikawa cells stably over-expressing β-catenin (Ish-bcat#5) on western blot and reverse transcription (RT)–PCR assays. (B) Ishikawa cells were transfected with 100 ng of pGL3–[−760/+261]-Cdx2-Luc, 200 ng of pcDNA3.1-β-catenin and 50 ng of pCI-p300, together with the pRL-TK plasmid (50 ng). The experiment was performed in triplicate. Relative activity was determined based on arbitrary light units of luciferase activity normalized for pRL-TK activity, and the activities of the reporter plus the effector relative to that of the reporter plus empty vector are shown as means ± SDs. (C) The various promoter deletion constructs used for evaluating transcriptional regulation of the Cdx2 promoter. Numbers for each construct correspond to the 5′- and 3′-bp locations within the promoter (relative to the transcription start site as +1). (D) Ishikawa cells were transfected with 100 ng of pGL3–[−760/+261] Cdx2-Luc, 200 ng of pcDNA3.1-β-catenin and 50 ng of pcDNA3.1-TCF4ΔN30 (dominant-negative form), together with the pRL-TK plasmid (50 ng). The experiment was performed in triplicate. (E) Ishikawa cells were transfected with 100 ng of pGL3–[−760/+261] Cdx2-Luc and various amounts (100 and 250 ng) of pcDNA3.1-Cdx2, together with the pRL-TK plasmid (50 ng). The experiment was performed in triplicate. (F and G) Ishikawa cells were transfected with 100 ng of the 5′-various deletion constructs and 250 ng of pcDNA3.1-β-catenin (F) or pcDNA3.1-Cdx2 (G), together with the pRL-TK plasmid (50 ng). The experiment was performed in triplicate.
HA. Fluorescein isothiocyanate- or rhodamine-labeled anti-mouse or rabbit IgG (Molecular Probes, Leiden, Netherlands) was used as secondary antibodies, as described previously (19–21).

Statistics
Comparative data were analyzed using the Mann–Whitney U-test and the Spearman correlation coefficient. The cut-off for statistical significance was set at $P < 0.05$.

Results
Immunohistochemistry and in situ hybridization findings
Examples of immunohistochemistry findings for $\beta$-catenin, Cdx2, p21$^{Waf1}$, p27$^{Kip1}$, total pRb and Ki-67, as well as in situ hybridization for Cdx2 mRNA, in Em Cas with morules, are illustrated in Figure 1. In addition to the observed immunoreactivity for $\beta$-catenin, p21$^{Waf1}$, p27$^{Kip1}$, total pRb and Ki-67, as described previously (19–21), Cdx2 immunoreactivity, along with increased mRNA signals, was frequently found in morular but not Sur-Ca lesions, showing an apparent overlap with nuclear $\beta$-catenin (Figure 1A). In contrast, p27$^{Kip1}$ immunoreactivity was diffusely distributed in tumor lesions in most cases (Figure 1B).

Average LI$s$ for nuclear $\beta$-catenin and p21$^{Waf1}$ were significantly higher in morules than Sur-Ca lesions, in contrast to Ki-67 LI$s$ and total pRb scores, as described previously (19–21). Significant high values for Cdx2 LI$s$ were also observed in morules, whereas p27$^{Kip1}$ LI$s$ did not differ between the two phenotypes (Figure 1C).

Nuclear $\beta$-catenin LI$s$ were positively correlated with LI$s$ for Cdx2 and p21$^{Waf1}$, and inversely to pRb LI$s$ and Ki-67 LI$s$. Similar correlations among Cdx2 LI$s$, Ki-67 LI$s$ and total pRb LI$s$ were also observed (Table I).

In normal endometrial tissues including epithelial and stromal components, Cdx2 immunoreactivity was never found during any phase of the menstrual cycle, whereas $\beta$-catenin immunoreactivity was limited to cell membrane, with the exception of the focal nuclear accumulation in epithelial elements in the secretory early phase (data not shown).

Transactivation of Cdx2 promoter by $\beta$-catenin and Cdx2
To address the mechanisms responsible for over-expression of Cdx2 in morular lesions, we first examined whether over-expression of $\beta$-catenin can induce Cdx2 expression. Ishikawa cells stably over-expressing an active form $\beta$-catenin (HA-tagged $\beta$-cat$^{Δ45}$) (Ish-bcat$^{Δ45}$) showed a significant increase in endogenous Cdx2 LI$s$ at both mRNA and protein levels, as compared with the mock-transfected case (Figure 2A). Cdx2 promoter activity was...
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Fig. 4. Hec251 cells stably over-express Cdx2. (A) Upper panel: western blot analysis results demonstrating over-expression of HA-Cdx2 in clones #117 and #130 but not the mock transfected cells. Lower panel: note the HA-Cdx2 expression in nuclei of clone #117 cells, as well as the mock-transfected cells, were seeded at low density (1 × 10⁴) and monitored for growth. Cell numbers are presented as means ± SDs. (C) Western blot analysis for expression of regulators for G1–S transition at different days of cell growth in Cdx2-stable cell lines (#117 and #130) and mock-transfected cells. (D) Reverse transcription (RT)–PCR analysis of HA-Cdx2 and p21Waf1 mRNA expression in the Cdx2-stable cell lines (#117 and #130) and mock-transfected cells. The HA-Cdx2 mRNA was detected using a combination of 5′-HA forward and Cdx2 mRNA partial reverse primers (supplementary Table S1 is available at Carcinogenesis Online). Primer sequence for 5′-HA is 5′-TACCCATACGATGTTCCAGATTACGC-3′. (E) Ishikawa cells were transfected with 100 ng of p21Waf1-Luc and various amounts (100 and 250 ng) of pcDNA3.1-HA-Cdx2, together with the pRL-TK plasmid (50 ng). The experiment was performed in triplicate. Relative activity was determined based on arbitrary light units of luciferase activity normalized for pRL-TK activity, and the activities of the reporter plus the effector relative to that of the reporter plus empty vector are shown as means ± SDs. (F) Actinomycin D (ActD) chase studies in Cdx2-stable cell line (#117) and mock-transfected cells. Cells were grown to 50% confluence and treated with 7.5 μg/ml actinomycin D. Total RNA was extracted at different times after actinomycin D treatment and analyzed by reverse transcription–PCR. p21Waf1 mRNA was normalized against GAPDH mRNA.

increased ~2.5-fold by transfection of β-catΔS45, the effect being further enhanced to 4.5-fold by cotransfection of the p300 coactivator (Figure 2B). A search of the Cdx2 promoter for potential TCF-binding site (5′TTTG T/A T/A) revealed the presence of two sites, at –426 to –420 bp and –226 to –220 bp (Figure 2C). However, β-catenin-dependent transactivation was not affected by cotransfection of dominant-negative form TCF4 (Figure 2D).

The sequence analysis also demonstrated three putative Cdx-responsive elements (TATA T/C) located at –554, –22 and +201 bp from the transcriptional start site (Figure 2C), indicating that Cdx2 expression may be regulated, in part, by Cdx2 itself. As expected, Cdx2 could activate its own promoter ~4- to 6-fold in a dose-dependent manner (Figure 2E).

To determine the sequence in the Cdx2 promoter involved in β-catenin and Cdx2 responsiveness, a series of 5′-truncated promoter constructs were generated (Figure 2C). Deletion from –760 to –39 bp had little effect on induction of promoter activity by β-catΔS45, but deletion of –39 to +9 bp resulted in decreased responsiveness (Figure 2F). Similar findings were also observed in the case of Cdx2 (Figure 2G), indicating that a region of the proximal promoter encompassing –39 to +9 bp might contain putative regulatory sites that are responsive to both β-catenin and Cdx2.

Knowing that both β-catenin and Cdx2 are able to bind indirectly or directly to the TATA box (28,29), two additional reporter constructs carrying mutations in either TATA box or CdxRE2 site, respectively, were generated (Figure 3A). The two mutants were still activated by β-catΔS45, in contrast to considerable decreased Cdx2 responsiveness. Mutations in the CdxRE2 motif also resulted in a reduction in the basal activity of ~75% (Figure 3B and C). Finally, ChIP assays revealed that the transfected Cdx2 promoter recruited Cdx2 to the proximal region (Figure 3D), strongly suggesting that the region is required for Cdx2 binding to the own promoter.

Inhibition of cell proliferation by over-expressing Cdx2

The finding of an inverse correlation between Cdx2 expression and cell proliferation in Em Ca tissues allows speculation that Cdx2 may be involved in regulation of Em Ca cell proliferation. To test this, we first established cell lines stably over-expressing HA-tagged Cdx2, using Hec251 cells with very low levels of endogenous Cdx2 expression. Nuclear accumulation of HA-tagged Cdx2 was diffusely...
observed in the two-independent stable clones #117 and #130 (Figure 4A), demonstrating significant decrease in the proliferation rate, particularly in the exponential growth phase, as compared with the mock-transfected case (Figure 4B), in contrast to the lack of significant changes in morphology. To examine whether Cdx2 affects expression of regulators of the restriction point at the G1–S transition, we performed western blot assays. Both stable lines showed considerable decrease in expression of total pRB and its phosphorylated forms, G1 cyclins (cyclin A and D1), p27Kip1 and p16INK4A (Figure 4C). In contrast, p21^{Waf1} expression was markedly increased at both protein and mRNA levels (Figure 4C and D), despite no changes in the promoter activity (Figure 4E). In addition, an actinomycin D chase study revealed the half-life of p21^{Waf1} mRNA to be significantly increased in the stable line (#117) as compared with the mock-transfected case (Figure 4F), suggesting post-transcriptional modulation.

**Inhibition of β-catenin/TCF4-mediated transcription by Cdx2**

To investigate potential effects of Cdx2 on β-catenin/TCF4 signaling in Em Ca cells, we first performed one-hybrid assays. pG5-Luc activity was significantly increased ~4.5-fold by cotransfection of Cdx2 with a DNA-BD-fused full-length fragment (pM-β-cat) and deletion of the C-terminus of β-catenin (pM-β-catΔC), in contrast to the relatively minor change with deletion of the N-terminus (pM-β-catΔN) (Figure 5A). However, transfected Cdx2 resulted in not only no recruitment to nuclear aggregates formed by over-expressing β-catenin (Figure 5B) but also an inhibition of a β-catenin/TCF4-mediated transcription, as assessed with reference to Top and Fop reporter constructs (Figure 5C), in line with the findings for transactivation of p14ARF and cyclin D1 genes, known as β-catenin/TCF4 transcriptional targets, although Cdx2 itself acted as a positive regulator for the former but not the latter (Figure 5D).

**Discussion**

Several lines of evidence from our present study support the conclusion that Cdx2 expression is under transcriptional control by nuclear β-catenin. First, nuclear accumulation of β-catenin significantly overlapped Cdx2 immunoreactivity in morular lesions of Em Ca tissues, with a very significant positive correlation (r = 0.84, P < 0.0001). In contrast, the discrepant results for Cdx2 expression and nuclear β-catenin in the early secretory phase may indicate the existence of a critical threshold of the latter in triggering transcription of Cdx2. Second, cells stably over-expressing active form β-catenin showed an increase in endogenous Cdx2 expression at both mRNA and protein levels. Third, the Cdx2 promoter was activated by transfection of β-catenin, through the proximal region (~39 to +9 bp), the effects being further enhanced by transcriptional coactivator p300, suggesting regulation at the transcriptional level. Although most known β-catenin target genes require TCF/LEF factors for their activation (30–32), the observed activation did not require the TCF/LEF site in the promoter, as evidenced by the failure of dominant-negative TCF4 to inhibit β-catenin-mediated transcription. Similar findings have also been reported with other genes, including LEF1, p16INK4A and PML (21,33,54).

A major function of β-catenin is recruit to the basal transcription machinery to promoter regions of target genes in the Wnt-signaling
pathway, and there is evidence that transactivating elements of β-catenin interact specifically and directly with TATA-binding protein (28). In our study, however, mutations of the TATA box in Cdx2 promoter did not abolish the β-catenin responsiveness, indicating that β-catenin may interact with other general transcription factors. In fact, β-catenin also regulates promoter-specific gene activation that is mediated by RNA polymerase II complexes (28,34).

In addition to nuclear β-catenin, we also demonstrated that Cdx2 could activate its own promoter through interaction with two AT-rich motifs (TATA box and CdxRE-2) within the proximal promoter, thereby forming an autoregulatory loop, in line with earlier reports (35,36). Moreover, mutations in the latter resulted in reduction of the basal activity, leading us to speculate that the motif may be also important for Cdx2 expression. In contrast, it was recently shown that p27Kip1 physically interacts with Cdx2 and promotes proteasome-dependent degradation of Cdx2 in intestinal epithelial growing cells, suggesting a regulation primarily related to protein stability (37). In our results, however, cells stably over-expressing Cdx2 showed a decrease in p27Kip1 expression, probably in response to the slow cell proliferation. In addition, p27Kip1 expression did not differ between murine and Sur-Ca areas within Em Cas, so there was no correlation with the Cdx2 status. Considering the evidence of increased Cdx2 mRNA signals in muroral lesions, it is conceivable that the transcriptional activation may occur during the transdifferentiation process.

Importantly, over-expression of Cdx2 caused up-regulation of p21Waf1 due to mRNA stabilization, resulting in a significantly lowered cell growth rate. There are various pieces of evidence that different agents can modulate stability of p21Waf1 mRNA though interaction with RNA-binding proteins and multiple 3′-untranslated region cis-elements (38,39) allowing us to speculate that Cdx2 may also function cooperatively with these proteins. Although transactivation of p21Waf1 via binding to its promoter by Cdx2 has been demonstrated in osteosarcoma and gastric and colorectal carcinoma cell lines (40), we failed to confirm such associations in Em Ca cells. Similar anomalous findings have also been demonstrated in InR1-G9 cells and BHK fibroblasts (36). In agreement with other reports (23,41), it is probable that Cdx2 may require some cell type-specific factors to regulate downstream target genes.

The β-catenin protein possesses multiple transactivating elements at its N- and C-termini, which can operate independently from TCFs (42). Based on the results of one-hybrid assays, we expected a possible interaction between Cdx2 and β-catenin terminal portions, in particular the N-terminus. However, transfected Cdx2 resulted in not only a lack of association with nuclear aggregated β-catenin but also an inhibition of transcriptional activation of β-catenin/TCF4 target genes, indicating that the over-expression may cause an inhibition of a formation of transcriptionally active complexes with TCF4. In addition, Cdx2 itself showed opposite effects with regard to transactivation of the p14ARF and cyclin D1 promoters. At the present time, although we are unable to provide an appropriate explanation for the observations, it appears that interactions with other transcriptional factors may contribute to promoter-specific mechanisms of action.

Together, our observations suggested a model for functional association between Cdx2 and nuclear β-catenin during transdifferentiation of Em Ca cells (Figure 5E). Over-expression of Cdx2 mediated by both a TCF4-independent β-catenin transactivation and its own autoregulatory loop thus could lead to up-regulation of p21Waf1 expression due to stabilization of the mRNA, modulating β-catenin/TCF4-mediated transcription through unknown factors (X), and in turn resulting in inhibition of cell proliferation. However, over-expression of Cdx2 alone was not sufficient for triggering of changes in cell morphology, suggesting that cooperation with β-catenin signaling may be required for the transdifferentiation process. In fact, over-expression of cyclin D1, a target gene of β-catenin/TCF4 complexes, is considered to be due to induction of squamous metaplasia in murine mammary epithelium (43).

In conclusion, the present study provided evidence that an association between Cdx2 and β-catenin signaling could clearly participate in the transdifferentiation process of Em Ca cells.

Supplementary material
Supplementary Table S1 can be found at http://carcin.oxfordjournals.org/

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References

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