

ORIGINAL ARTICLE

# The intestine-specific homeobox gene *Cdx2* decreases mobility and antagonizes dissemination of colon cancer cells

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**The gravity of colorectal cancer is mainly due to the capacity of tumor cells to migrate out of the tumor mass to invade the stroma and disseminate as metastases. The acquisition of a migratory phenotype also occurs during wound healing. Here, we show that several features characterizing invasive colon tumor cells are shared by migrating cells during wound repair *in vitro*. In particular, the expression of the intestine-specific transcription factor *Cdx2*, a key gene for intestinal identity downregulated in invasive colon cells, is reduced during wound healing *in vitro*. Transcription factors involved in epithelial–mesenchymal transition such as Snail and Slug are upregulated during wound healing and are able to repress *Cdx2* transcription. *In vitro*, forced expression of *Cdx2* in human colon cancer cell lines retarded wound repair and reduced migration, whereas inhibition of *Cdx2* expression by RNA interference enhanced migration. *In vivo*, forced expression of *Cdx2* opposed tumor cells spreading in nude mice xenografted at three different sites. These data provide evidence that *Cdx2* antagonizes the process of tumor cell dissemination, and they suggest that this homeobox gene might represent a new therapeutic target against metastatic spreading of colon cancer.**

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**Keywords:** Cdx; colon cancer; metastasis; migration; wound healing

## Introduction

Colorectal cancer (CRC) is a major problem of public health because of its incidence and gravity. Gravity is mainly due to the propensity of malignant cells to escape the primary tumor and form metastases. The progression from adenoma to metastases is accompanied by an accumulation of irreversible genomic alterations (Diep *et al.*, 2006). Dissemination

of malignant cells from the tumor mass into the stroma is also characterized by reversible changes in gene and protein expression that mark the ability of invasive cells to adapt and migrate into the stroma (Brabletz *et al.*, 2005). Reversible nuclear translocation of  $\beta$ -catenin is a hallmark of invasive CRC cells, leading to transient changes of downstream targets, including metalloproteinases and adhesion molecules (Brabletz *et al.*, 2005; Gavert *et al.*, 2005). Invasive regions of CRC are characterized by the loss of basement membrane and by the activation of transcription factors related to the process of epithelial–mesenchymal transition (EMT), which is highly relevant for the presence of distant metastases and overall survival (Conacci-Sorrell *et al.*, 2003; Franci *et al.*, 2006; Spaderna *et al.*, 2006).

Recently, we reported that the homeobox gene *Cdx2* is also transiently decreased in invasive cells at the tumor/stroma interface of differentiated CRC (Brabletz *et al.*, 2004). *Cdx2* encodes a transcription factor that plays a crucial role in the determination of intestinal identity during embryogenesis (Beck *et al.*, 1999). Its expression is specifically retained in the intestinal epithelium throughout adulthood, and becomes heterogeneous and reduced in CRC (Ee *et al.*, 1995; Kaimaktchiev *et al.*, 2004; Subtil *et al.*, 2007). The relevance of the *Cdx2* decline has been demonstrated since reduced levels in *Cdx2*<sup>+/-</sup> mice facilitates colon tumor progression (Aoki *et al.*, 2003; Bonhomme *et al.*, 2003). However, the functional consequences of the selective decline of *Cdx2* in invasive cells have not been addressed.

Similarities between cancer and wound healing led to consider cancers as wounds that do not heal (Dvorak, 1986). Whole-genome microarray analyses provided molecular support for this concept, as illustrated by common gene expression signatures (Pedersen *et al.*, 2003; Chang *et al.*, 2004). Importantly, a wound healing signature in tumor specimens correlates with the reduction of overall survival and distant metastasis-free survival (Chang *et al.*, 2004, 2005).

Here, we found that wound repair *in vitro* is accompanied by a decrease of *Cdx2* expression and we provide *in vitro* and *in vivo* evidence showing that *Cdx2* opposes colon cancer cell migration and dissemination.

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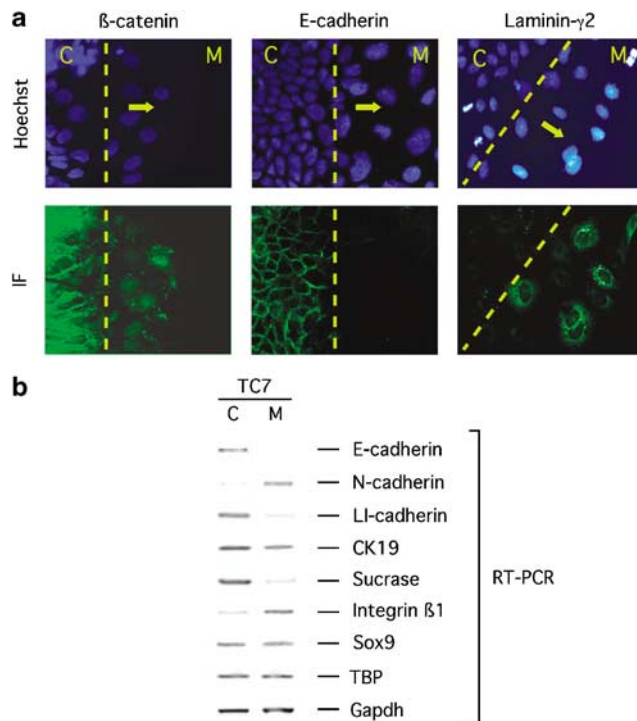
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## Results

### *Migrating cells during wound repair in culture share molecular properties with invasive CRC cells*

We examined in the wound healing model the expression and/or protein distribution of molecules known to be modified in invasive CRC cells. For this, human colon cancer cells Caco-2/TC7, which undergo spontaneous enterocytic differentiation (Chantret *et al.*, 1994), were grown to confluence and then the monolayer was scraped. As already described (Pandrea *et al.*, 2000), after 24–48 h cells at the wound edge exhibited a flat morphology compared to the adjacent monolayer (Supplementary Figure 1). Immunocytofluorescence analysis performed at 48 h indicated that (i)  $\beta$ -catenin was membranous in confluent cells, but nuclear in migrating cells of the wound edge, (ii) E-cadherin exhibited a reduced and fuzzy expression in migrating cells and (iii) laminin- $\gamma$ 2 accumulated intracellularly in migrating cells (Figure 1a). Interestingly, similar modifications have been reported in invasive CRC cells (Cavallaro and Christofori, 2004; Brabletz *et al.*, 2005).

Next, we performed reverse transcription (RT)–PCR with RNA extracted from cells of the wound edge and of the confluent monolayer (Figure 1b). Invasive cancer

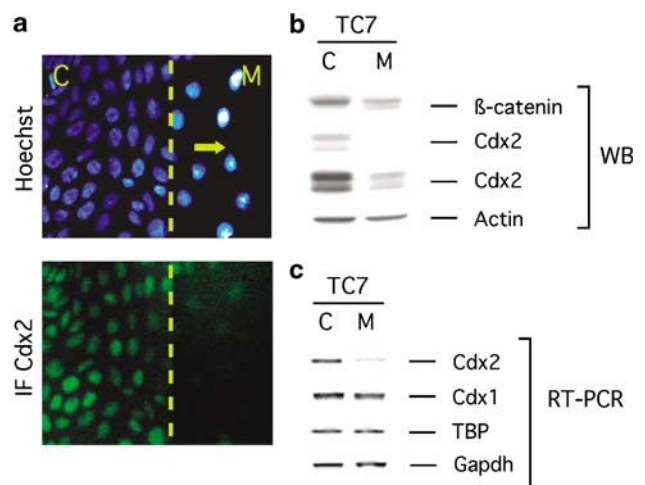


**Figure 1** Expression changes during the wound repair in Caco-2/TC7 cells. **(a)** Confluent Caco-2/TC7 were wounded and left for 48 h before immunofluorescence (IF) detection of  $\beta$ -catenin, E-cadherin and laminin- $\gamma$ 2. Cell nuclei were stained with Hoechst. The dotted line corresponds to the wound and the arrow indicates the sense of migration. C, confluent monolayer; M, migrating cells at the wound edge. **(b)** At 48 h after wounding, cells at the wound edge (M) and of the confluent monolayer (C) were separately collected for RNA extraction. Expression of the indicated genes was analysed by reverse transcription (RT)–PCR. Gapdh and TBP were used as internal controls.

cells often show a cadherin switch during which E-cadherin is reduced at the benefit of N-cadherin (Hazan *et al.*, 2004). During wound healing, migrating cells similarly exhibited decreased E-cadherin and increased N-cadherin mRNA levels. The LI-cadherin transcripts were also reduced in migrating cells, whereas those of integrin- $\beta$ 1 were increased. mRNA level of sucrase-isomaltase, an apical digestive enzyme, was also reduced in migrating cells. In contrast, the transcripts of cytokeratin-19 and Sox9 were unchanged and vimentin mRNA was undetected in migrating and confluent cells.

### *Cdx2 expression is reduced in migrating cells at the wound edge*

The sucrase-isomaltase and LI-cadherin genes, down-regulated in migrating cells of the wound edge, are direct targets of the transcription factor Cdx2 (Suh *et al.*, 1994; Hinoi *et al.*, 2002). Therefore, we analysed Cdx2 in the wound healing model (Figure 2). Immunocytofluorescence staining showed that Cdx2 protein accumulated in cell nuclei of the confluent monolayer, but was reduced in cells of the migrating front. Corroborating these data, RT–PCR and western blots on cellular extracts of the confluent monolayer and migrating front revealed less Cdx2 mRNA and protein in migrating cells, without any detectable change in phosphorylated isoforms of Cdx2, as assessed by highly exposed western blots. In contrast, the weak Cdx1 expression was unchanged in migrating



**Figure 2** Downregulation of Cdx2 expression during *in vitro* wound healing. **(a)** Confluent Caco-2/TC7 were wounded and left for 48 h before immunofluorescence detection of Cdx2. Hoechst staining visualizes the cell nuclei. **(b)** At 48 h after wounding, cells of the wound edge (M) and of the confluent monolayer (C) were separately collected for protein extraction. Expression of the indicated proteins was analysed by western blot. Short and long exposures are shown for Cdx2. **(c)** Cells were collected as described above **(b)** for RNA analysis by reverse transcription (RT)–PCR. Of note, Cdx1 mRNA is expressed at a much lower level in Caco-2/TC7 cells than the Cdx2 transcript, as exemplified by the fact that RT–PCR needs 22 amplification cycles for Cdx2, but 36 cycles to get the band illustrated here for Cdx1. In line with this, the Cdx1 protein could not be analysed by western blot in these experiments because the endogenous expression of this protein in Caco-2/TC7 cells was below the level of detection.

cells. These data provide evidence of a decreased expression of *Cdx2* in human colon cancer cells committed to migrate *in vitro*. Most interestingly, a specific decrease of this transcription factor also occurs in the invasive CRC cells (Brabletz *et al.*, 2004). Thus, the wound healing model recapitulates the changes observed for *Cdx2* in invasive cells and is appropriate to address the cellular consequences of these changes.

*Cdx2* expression is sensitive to EMT-inducing transcription factors

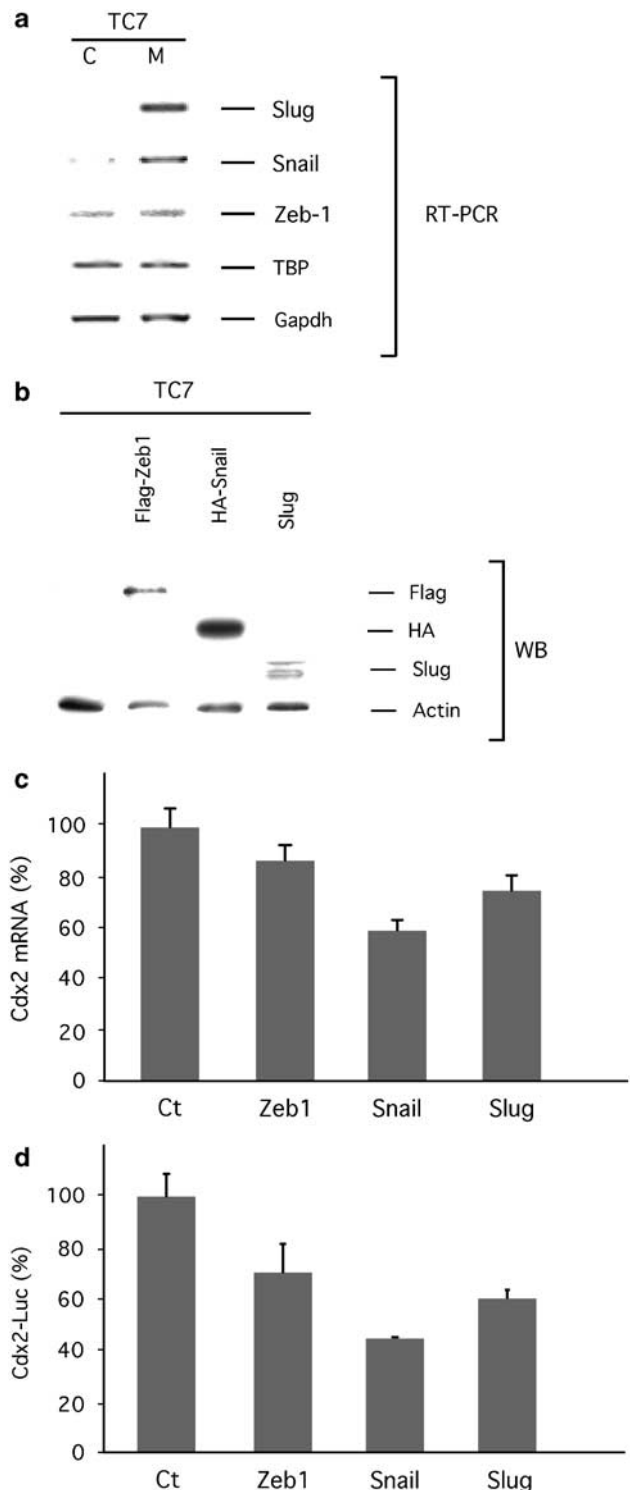
Epithelial cells at the wound edge are migrating, like mesenchymal cells. Thus, we determined if transcription factors involved in EMT can downregulate *Cdx2*. First, we analysed the mRNA expression of *Snail*, *Slug*, *Twist*, *Zeb1* and *Zeb2* in Caco2/TC7 cells subjected to wound healing (Figure 3a). RT-PCR indicated that *Snail* and *Slug* mRNA were barely detected in confluent cells, but strongly increased in migrating cells at the wound edge. Unlike *Snail* and *Slug*, *Zeb1* mRNA was already expressed in confluent cells and only slightly augmented in migrating cells. Both *Twist* and *Zeb2* mRNA remained undetectable.

Next, *Zeb1*, *Snail* and *Slug* were overexpressed in Caco-2/TC7 cells by transient transfection (Figure 3b), and endogenous *Cdx2* expression was checked by RT-qPCR. All these repressors downregulated *Cdx2* and *Snail* was the most effective with a 40% reduction in *Cdx2* mRNA (Figure 3c). Then, we tested the effects of these repressors on the *Cdx2* promoter by cotransfection with the luciferase reporter plasmid pCdx2-1Luc (Lorentz *et al.*, 1999). Corroborating the RT-qPCR results, overexpression of *Zeb1*, *Snail* or *Slug* reduced the activity of the promoter, with *Snail* showing the highest effect (Figure 3d). Of note, the extent of repression of the *Cdx2* promoter was comparable to that found with the *E-cadherin* promoter, a typical target of *Snail* (not shown). Thus, transcription repressors associated to EMT, in particular *Snail* and *Slug*, are stimulated in migrating Caco2/TC7 cells and can downregulate *Cdx2*.

Inverse correlation between *Cdx2* levels and cell migration capacity in vitro

Having shown that *Cdx2* expression decreases in cells of the wound edge, we asked if this had an effect on the

repair process using a gain-of-function approach. Stable cell lines expressing *Cdx2* upon doxycycline treatment (see Supplementary Information and Supplementary Figure 2 for details) were established from human colon cancer cells HT29-16E/TR that contain a doxycycline-inducible system and express only trace amounts of endogenous *Cdx2* in culture (Blache *et al.*, 2004). Control and *Cdx2*-inducible clones were grown to



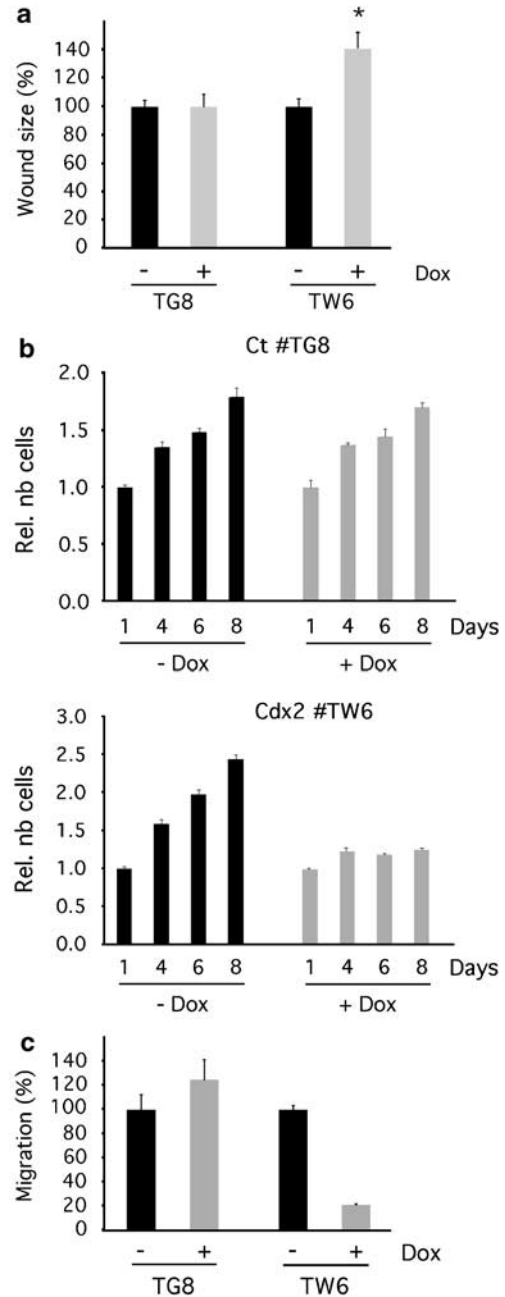
**Figure 3** Repression of *Cdx2* by epithelial-mesenchymal transition (EMT)-inducers in Caco-2/TC7 cells. **(a)** Caco-2/TC7 cells of the wound edge (M) and confluent layer (C) were analysed for RNA expression of the indicated genes by reverse transcription (RT)-PCR. **(b** and **c**) Caco-2/TC7 cells were transfected with plasmids expressing *Zeb1*, *Snail* or *Slug* for 48 h before isolation of the transfected cells with MACSelect and protein or RNA extraction. Expression of exogenous *Zeb1*, *Snail* and *Slug* was checked by western blot **(b)** and expression of *Cdx2* ( $\pm$ s.d.; set at 100% in the absence of the repressors) was analysed by real-time RT-qPCR **(c)**. **(d)** Caco-2/TC7 cells were cotransfected with pCdx2-1Luc and expression plasmids for *Zeb1*, *Snail* or *Slug* for 48 h before luciferase assay. Data correspond to a representative experiment performed in triplicate. Expression ( $\pm$ s.d.) of the pCdx2-1Luc reporter in the absence of the repressors was set at 100%.

confluence and a wound was performed in absence or presence of doxycycline in the culture medium. The dimension of the wound was measured 48 h later (Figure 4a). The wound size was independent of doxycycline treatment in control cells. In contrast, turning on Cdx2 expression by doxycycline in Cdx2-inducible cells resulted in a larger wound compared to untreated cells. Thus, Cdx2 expression antagonizes wound repair.

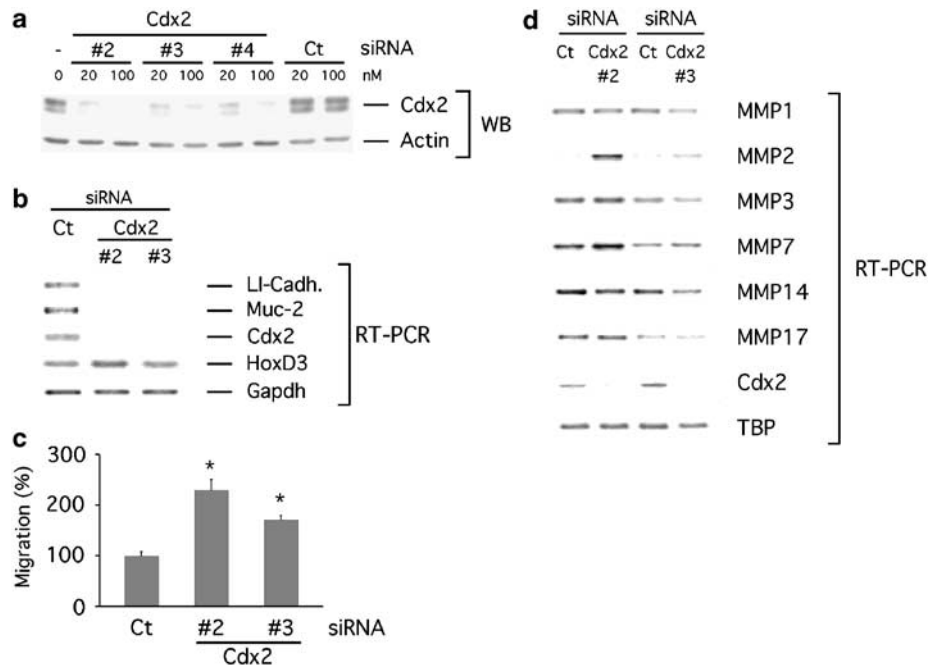
Since wound healing involves proliferation and migration, we addressed the impact of Cdx2 on both aspects. First, proliferation was examined using the MTS assay. In subconfluent control clones, the number of cells regularly increased during culture, independently of doxycycline (Figure 4b, top panel). In subconfluent Cdx2-inducible clones, the number of cells also regularly increased without doxycycline; however, cell growth was impaired upon Cdx2 induction by doxycycline, as cell number increased very slowly (Figure 4b, bottom panel). Immunofluorescence analysis with anti-phospho-histone H3 antibody confirmed the reduction of cell proliferation, which occurred without significant apoptosis, as shown by active caspase-3 immunostaining (Supplementary Figures 3a and b).

Next, we investigated whether forced Cdx2 expression had an effect on cell migration, using Boyden chambers in which cells are migrating through a porous membrane independently of cell proliferation (Figure 4c). For control clones, the number of cells having migrated through the filter after 24 h was not decreased in doxycycline-treated compared to -untreated cells. In contrast, for the Cdx2-inducible clones, the number of cells having migrated through the filter was reduced up to fivefold in the presence of doxycycline, indicating that Cdx2 expression decreases cell migration.

Conversely, we asked if decreasing Cdx2 expression experimentally in cancer cells had an impact on their migration capacity, given that Cdx2 is downregulated in migrating cells during *in vitro* epithelial repair and local invasion in CRC. For this, we developed a loss-of-function approach using short interfering RNA (siRNA)@Cdx2. When transfected into human colon cancer SW480 cells, which endogenously express *Cdx2*, and to a much lower extent *Cdx1* (data not shown), siRNA@Cdx2 reduced Cdx2 protein expression dose-dependently (Figure 5a). Of note, the siRNA@Cdx2#2 specifically inhibited *Cdx2*, whereas the siRNA@Cdx2#3 inhibited both *Cdx2* and *Cdx1* (Supplementary Figure 4). None of them inhibited the expression of another homeobox gene, *HoxD3* (Figure 5b). Along with *Cdx2*, two of its targets, *LI-cadherin* and *Muc2* (Hinoi et al., 2002; Mesquita et al., 2003), were also strongly decreased by siRNA@Cdx2 (Figure 5b), attesting the functional relevance of the Cdx2 decline by this approach. To check migration, SW480 cells transfected with siRNA@Cdx2 or with a control siRNA were harvested and seeded into Boyden chambers. Figure 5c shows that cells migrated about twofold more efficiently through the pores when transfected with either siRNA@Cdx2 compared to control siRNA. Thus, there is an inverse



**Figure 4** Forced Cdx2 expression inhibits proliferation and migration *in vitro*. (a) Monolayers of control (TG8) or Cdx2-inducible (TW6) cells were wounded with a pipet tip and left for 48 h with/without doxycycline. The size of the wound in the absence of doxycycline was set at 100% for each clone to quantify the data. s.d. corresponds to s.d. out of nine measurements (three separate wounds measured in three different areas). Asterisk indicates significant difference ( $P < 0.01$ ). (b) Control and Cdx2-inducible cells were grown with/without doxycycline and the number of cells was quantified at the indicated day post-seeding using a MTS proliferation assay. The value ( $\pm$  s.d. out of six separate wells) obtained 1 day after seeding for each clone was set at one for quantification. (c) Control and Cdx2-inducible cells were seeded on the top of a porous filter in the growth medium deprived of serum, with/without doxycycline. After 24 h, cells having migrated through the filter towards the growth medium (10% fetal bovine serum) supplemented with 100 nM phorbol myristate acetate, were fixed and stained. Cells were counted in five areas for each filter and results ( $\pm$  s.d.) of representative experiments performed in triplicates are shown.



**Figure 5** Reduced Cdx2 expression stimulates migration *in vitro*. (a) SW480 cells were transfected for 3 days with the different short interfering RNA (siRNA) before immunoblotting with the indicated antibodies. (b) As in (a), but with gene expression being analysed by reverse transcription (RT)-PCR. (c) SW480 cells having migrated through the porous membrane after transfection of 20 nM siRNA@Cdx2 #2, siRNA@Cdx2 #3 or control siRNA were counted. Data are given  $\pm$  s.d. for three independent assays. Asterisks indicate significant difference ( $P < 0.01$ ). (d) As in (b).

correlation between Cdx2 expression and cell migration capacity.

#### Reduction of cancer cell dissemination upon forced Cdx2 expression *in vivo*

Since Cdx2 expression is reduced in migrating cells and that this decline enhances migration *in vitro*, we examined whether Cdx2 had an impact on tumor cell dissemination *in vivo*. For this, we xenografted Cdx2-inducible cells at three sites in nude mice: the cecum wall to investigate local invasion, the tail vein to follow-up dissemination through the lymphatic system, and the spleen to address liver homing. Each of the grafting models used 10 mice, five of them receiving drinking water containing doxycycline and the five others receiving doxycycline-free water. Mice were killed 13–16 weeks later for histopathological examination. Results are illustrated in Figure 6 and recapitulated in Table 1.

Upon grafting into the cecum wall, tumor cells were recovered in mesenteric lymph nodes in 5/5 mice without doxycycline, whereas invaded nodes only occurred in 2/5 mice with doxycycline administration. In both cases, 2/5 mice presented metastatic dissemination into the liver. Yet, only one tumor nodule was observed in the liver of each doxycycline-treated animal, whereas untreated mice showed respectively three and four nodules in their liver.

Tumor cell injection in the tail vein caused dissemination along the dorsal lymphocentral chain. Upon doxycycline treatment, only 1/5 mice exhibited lymph node invasion, which was restricted to one node of the posterior ileosacralis region and two nodes of the

midlumbalis region. Untreated mice showed more prominent spreading in lymph nodes. Indeed, 4/5 mice exhibited several invaded lymph nodes along the dorsal chain, reaching nodes of the anterior thoracic region in two mice. This suggested that doxycycline-induced Cdx2 expression reduced the capacity of tumor cells to progress anteriorly along the lymph node chain.

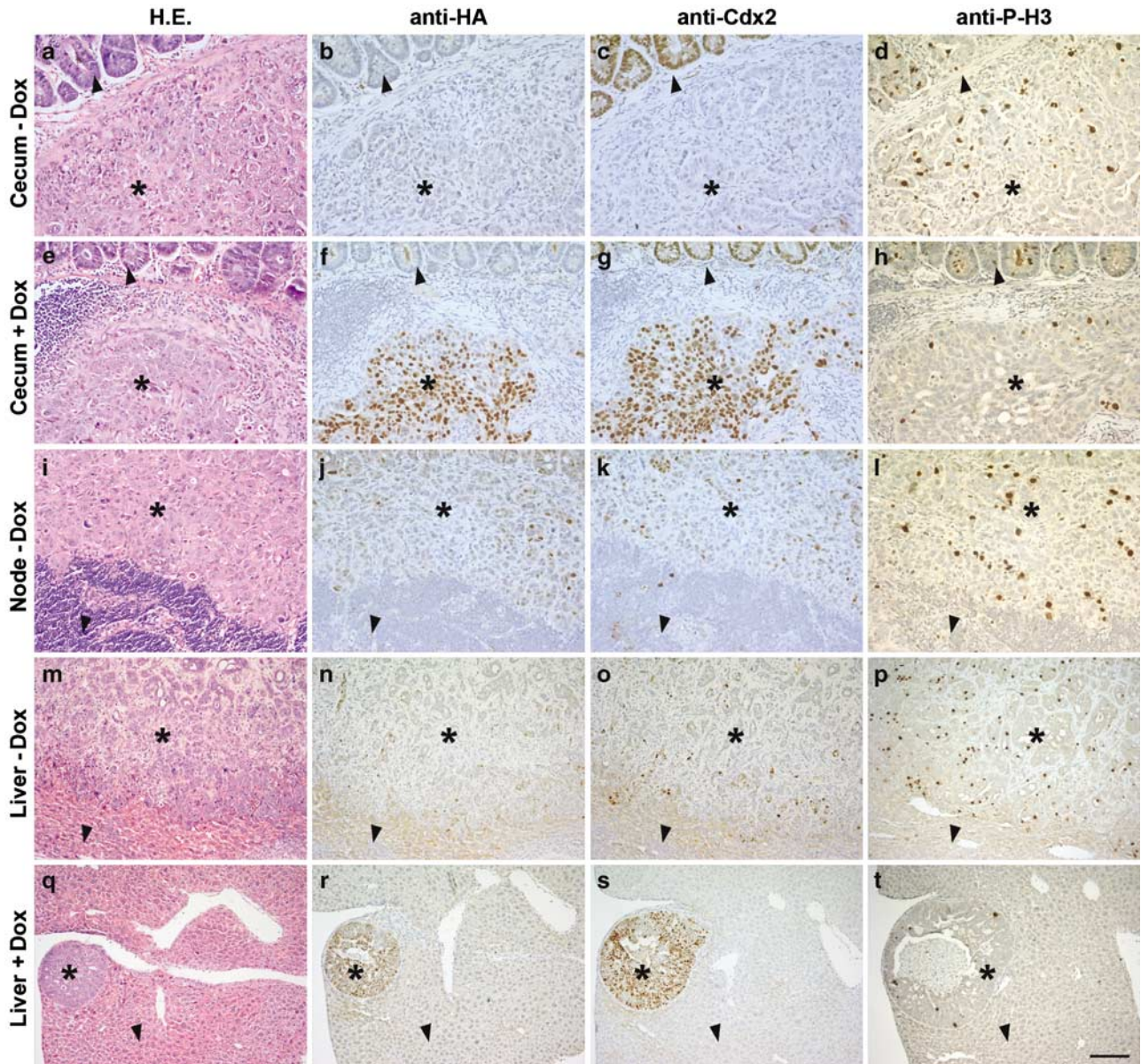
Upon injection of tumor cells in the spleen, 2/5 mice treated with doxycycline showed spreading into the liver, whereas spreading occurred in 5/5 untreated mice. In addition, 3/5 of the untreated mice exhibited invasion in the lymph nodes of the thoracic and lumbalis regions, whereas this did not occur in doxycycline-treated mice.

Phospho-histone H3 immunostaining in tumors revealed that cell proliferation was reduced upon Cdx2 induction (Figure 6). Apoptosis assessed by active Caspase-3 staining was low, irrespective of the level of Cdx2 (data not shown).

Altogether, these data indicate that Cdx2 expression inversely correlates with cell dissemination, suggesting that Cdx2 slows down cell spreading *in vivo*. Providing the prominent role of metalloproteinases in this process, we analysed their expression in SW480 cells upon siRNA-mediated inhibition of Cdx2 and found a specific increase of MMP2 mRNA (Figure 5d).

#### Discussion

The intestinal epithelium is highly organized with tightly linked polarized columnar cells. However, these cells



**Figure 6** Forced Cdx2 expression limits cancer cell dissemination *in vivo*. Cdx2-inducible cells (TW6) were injected in the cecum wall (a–l), the tail vein (not illustrated) and the spleen (m–t) of nude mice and the animals received drinking water with/without doxycycline, as indicated. Primary tumors growing in the cecum wall (a–d) and (e–h), a mesenteric lymph node metastase (i–l) and tumors spreading into the liver (m–p) and (q–t) were analysed by HE staining for histology and by immunohistochemistry with anti-HA, anti-Cdx2 and anti phospho-histone H3 (P-H3) antibodies. The mesenteric lymph node in (i–l) was from the mouse whose primary intra-cecal tumor is shown in (a–d). Asterisks indicate tumor cells and arrowheads denote host tissue: the epithelium of the cecum (a–h), the lymph node (i–l) and the liver parenchyma (m–t). Bar is 20  $\mu\text{m}$  in (a–l) and 40  $\mu\text{m}$  in (m–t).

exhibit plasticity as they can flatten and migrate in case of injury or cancer. Here, using a series of candidate genes and proteins, we found similar changes in mRNA expression and/or protein distribution between wound healing and invasive CRC cells. Changes concern widely expressed proteins ( $\beta$ -catenin, E- and N-cadherins, integrin- $\beta$ 1, laminin- $\gamma$ 2, Snail and Slug) and proteins more specific of the gut (sucrase-isomaltase, LI-cadherin). Consequently, we investigated the intestine-specific transcription factor Cdx2 and found a decreased expression in cells of the wound edge, as

reported previously in invasive CRC cells (Brabletz *et al.*, 2004).

In an attempt to delineate the cellular consequences of these changes, we used gain- and loss-of-function approaches to demonstrate that Cdx2 opposes cell migration *in vitro*. Moreover, gain-of-function showed that Cdx2 opposes tumor cells spreading in nude mice xenografted and injected at three different sites. These results provide evidence for an anti-metastatic role of Cdx2 and are of high significance because of the reduction of Cdx2 expression occurring in invasive

**Table 1** Effect of Cdx2 on tumor cell dissemination

	<i>No of animals with invaded mesenteric lymph nodes</i>	<i>No of animals with liver invasion</i>	<i>No of tumor nodules per liver</i>
Cecum			
–Dox ( <i>n</i> = 5)	5/5	2/2	3;4
+Dox ( <i>n</i> = 5)	2/5	2/2	1;1
	<i>No of animals with invaded lymphocentric nodes</i>	<i>Position of the anterior-most invaded lymph nodes</i>	
Tail vein			
–Dox ( <i>n</i> = 5)	4/5	Anterior thoracic region	
+Dox ( <i>n</i> = 5)	1/5	Mid-lumbalis region	
	<i>No of animals with invaded liver</i>	<i>No of animals with invaded lymphocentric nodes</i>	
Spleen			
–Dox ( <i>n</i> = 5)	5/5	3/5	
+Dox ( <i>n</i> = 5)	2/5	0/5	

TW6 colon cancer cells were injected in the cecum, the tail vein or the spleen of nude mice (10 mice each group). Three days later and throughout the duration of the experiment, five mice in each group received Doxycycline (+Dox) in drinking water and five mice received Doxycycline-free water (–Dox). Tumor development was analysed 16 weeks after cell injection in the cecum and 13 weeks after cell injection in the tail vein or the spleen. *n*, number of animals.

CRC cells (Brabletz *et al.*, 2004). Altered expression of homeobox genes has been reported in several cancers, leading to changes in cell migration and spreading. *HoxD10* impairs breast tumor cells migration (Carrio *et al.*, 2005). Inversely, *HoxD3* increases cell mobility and wound repair, while *HoxB13* and *Gooseoid* promote invasion (Hansen *et al.*, 2003; Zhao *et al.*, 2005; Hartwell *et al.*, 2006; Ohta *et al.*, 2006). The cell spreading effect of *HoxD3* correlates with its role in regulating cell–cell and cell–matrix interactions via the stimulation of integrin- $\alpha_V\beta_3$  and reduction of E-cadherin (Hamada *et al.*, 2001; Ohta *et al.*, 2006). Cdx2 plays a key role in intestinal differentiation by upregulating intestinal-specific genes and inhibiting cell proliferation, which may contribute to antagonize cancer cell dissemination. In addition, the inhibitory effect of Cdx2 on cell migration and spreading is in line with the fact that it controls the expression of extracellular matrix molecules and stimulates *LI-cadherin* and *E-cadherin* levels (Lorentz *et al.*, 1997; Hinoi *et al.*, 2002; Keller *et al.*, 2004). Moreover, we showed here that the decline of Cdx2 correlates with increased amounts of integrin- $\beta_1$  and the metalloproteinase MMP2, two molecules involved in tumor cell invasion. Thus Cdx2 may exert its inhibitory effect on colon cancer cell migration and spreading by acting on multiple cellular functions. At the molecular level, Cdx2 acts as a transcriptional activator as exemplified by the *sucrase-isomaltase*, *LI-cadherin* and *Muc2* genes (Suh *et al.*, 1994; Hinoi *et al.*, 2002; Mesquita *et al.*, 2003). However, we have shown that it can also inhibit the transcriptional activity of another homeodomain protein, Cdx1 (Gautier-Stein *et al.*, 2003; Calon *et al.*, 2007). Further experiments will address if the effect of Cdx2 on tumor cell migration and dissemination is linked to its transcriptional stimulatory or inhibitory activity and/or to the combination of these activities.

Reduced Cdx2 expression accelerates CRC progression (Aoki *et al.*, 2003; Bonhomme *et al.*, 2003), and the present study further indicates that the Cdx2 decline

facilitates tumor cell migration and spreading. Of note, the decline of Cdx2 in invasive cells is reversible (Brabletz *et al.*, 2004), indicating the involvement of downregulatory mechanism(s). Corroborating this conclusion, the *Cdx2* gene is neither mutated nor lost in CRC (Yagi *et al.*, 1999; Subtil *et al.*, 2007) while evidence exists for a dominant repressive mechanism silencing *Cdx2* in cancer cells (Hinoi *et al.*, 2003). However this mechanism remains elusive, in particular during wound healing and tumor cell invasion. Given that constitutively active  $\beta$ -catenin does not directly regulate the *Cdx2* promoter (Domon-Dell and Freund, 2002) and that Sox9, a target of  $\beta$ -catenin/TCF4 that inhibits *Cdx2* (Blache *et al.*, 2004), is not upregulated during *in vitro* wound healing, the decline of Cdx2 in migrating cells may not depend on  $\beta$ -catenin. We reported previously that *Cdx2* expression depends on the microenvironment and is downregulated by collagen type I via integrin- $\beta_1$  and FAK (Brabletz *et al.*, 2004; Benahmed *et al.*, 2007). Interestingly, we show here that Integrin- $\beta_1$  is upregulated during wound healing. Besides, we found that *Cdx2* expression is also sensitive to EMT-inducing transcription factors such as Snail and Slug. The *Cdx2* promoter harbors a canonical E-box (CAGCTG, around –400 pb), which might serve as a binding site for Snail or Slug but mutation of this site does not abolish the repression by Snail while results with Slug are highly variable and inconclusive (data not shown). Thus, the molecular mechanism of the Cdx2 decline in migrating cells, in particular the effect of Snail and Slug and the crosstalk with Integrin- $\beta_1$ /FAK signaling, remains to be elucidated.

Based on the above results, we propose that preventing the Cdx2 decline by counteracting the underlying downregulatory mechanism(s) may be a way to specifically slow-down CRC progression and metastatic spreading. Future experiments will deal with the identification of these mechanisms and with the search of compounds able to blunt this inhibitory action, to develop new therapeutic agents against CRC.

## Materials and methods

### Wound healing assay

Caco-2/TC7 cells ( $3 \times 10^6$ ) were seeded in 60 cm<sup>2</sup> dishes (RT-PCR or western blot) or  $5 \times 10^4$  in eight-well chamber slides (Lab-Tek, immunofluorescence) and grown to confluence. After 5 days, the monolayer was wounded with a pipet tip and cells detached upon wounding were carefully rinsed off. At 48 h after the wound, cells were collected with a scalpel under a binocular for RT-PCR or western blot, or fixed for immunofluorescence. Similar results were obtained at least thrice in independent experiments.

About  $12 \times 10^6$  control or Cdx2-inducible HT29-16E/TR derived cells (Supplementary Data) were seeded at confluence in 21 cm<sup>2</sup> dishes in the absence or presence of doxycycline and the monolayer was wounded the next day (three wounds per dish). After 48 h of incubation in growth medium supplemented with 100 nM phorbol myristate acetate (PMA) (Sigma, St Quentin, Fallavier, France, -/+ -doxycycline), three pictures of different areas of each wound were taken under a bright field microscopy and measurements were done thrice for each area (27 measurements per plate). The experiment was performed thrice with similar results, using two control and two Cdx2-inducible clones.

### Migration assay in Boyden chambers

Polyester membrane inserts were used to assess cell migration *in vitro* (8  $\mu$ m pore size, 6.5 mm membrane diameter; BD Biosciences, San Jose, CA, USA). Cells were harvested by trypsinization 2 days after doxycycline treatment or 3 days after siRNA transfection, counted, resuspended in 200  $\mu$ l serum-free medium with/without doxycycline ( $5 \times 10^3$  cells/ml) and seeded on the insert. The lower chamber contained 300  $\mu$ l of growth medium. For the inducible cells, the medium eventually contained doxycycline and was supplemented with 100 nM PMA (Sigma) since parental HT29 cells exhibit a poor intrinsic migration capacity (Mallo *et al.*, 1998). After 24 h, cells having migrated through the filter were fixed with methanol, stained with crystal violet (0.005%) and counted

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under a microscope in five randomly chosen fields. Similar results were obtained in three independent experiments performed in triplicate, using two control and two Cdx2-inducible clones.

### Xenografts

Male nu/nu mice, aged 6–8 weeks (Charles River), were housed under pathogen-free conditions. They were anaesthetized with isoflurane gas (Forene, Abott) at the time of surgery. For each mouse,  $1 \times 10^6$  HT29-16E/TR TW6 cells suspended in 50  $\mu$ l DMEM were injected with a 30G needle into the cecum wall (10 mice), or the spleen (10 mice) or the tail vein (10 mice). Starting 3 days later, five mice of each group received 400  $\mu$ g/ml doxycycline in drinking water throughout the experiment (fresh doxycycline-containing water every 2 days), whereas the other five received doxycycline-free water. Mice injected in the spleen and in the tail vein were killed 13 weeks after injection and mice injected in the cecum were killed after 16 weeks. Each mouse was carefully checked for the presence of tumors in the cecum, lymph nodes, abdominal cavity, liver and lung. Tumor samples were analysed by immunohistochemistry.

*Cell lines, proliferation assay, plasmids, siRNA, transfection, RT-PCR, western blot, immunohistochemistry*  
See Supplementary Information.

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Supplementary Information accompanies the paper on the Oncogene website (<http://www.nature.com/onc>).