Defective Claudin-7 Regulation by Tcf-4 and Sox-9 Disrupts the Polarity and Increases the Tumorigenicity of Colorectal Cancer Cells

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Abstract

Tight junctions have recently emerged as essential signaling regulators of proliferation and differentiation in epithelial tissues. Here, we aimed to identify the factors regulating claudin-7 expression in the colon, and analyzed the consequences of claudin-7 overexpression in colorectal carcinoma (CRC). In healthy human colonic crypts, claudin-7 expression was found to be low in the stem/progenitor cell compartment, where Tcf-4 activity is high, but strong in differentiated and postmitotic cells, where Tcf-4 is inactive. In contrast, claudin-7 was overexpressed in areas with high Tcf-4 target gene levels in CRC samples. *In vitro*, Tcf-4 was able to repress claudin-7 expression, and the high mobility group–box transcription factor Sox-9 was identified as an essential mediator of this effect. Claudin-7 was strongly expressed in the intestine of Sox-9–deficient mice and in CRC cells with low Sox transcriptional activity. Sox-9 overexpression in these cells reinstated claudin-7 repression, and residual claudin-7 was no longer localized along the basolateral membrane, but was instead restricted to tight junctions. Using HT-29Cl.16E CRC cell spheroids, we found that Sox-9–induced polarization was completely reversed after virus-mediated claudin-7 overexpression. Claudin-7 overexpression in this context increased Tcf-4 target gene expression, proliferation, and tumorigenicity after injection in nude mice. Our results indicate that Tcf-4 maintains low levels of claudin-7 at the bottom of colonic crypts, acting via Sox-9. This negative regulation seems to be defective in CRC, possibly due to decreased Sox-9 activity, and the resulting claudin-7 overexpression promotes a loss of tumor cell polarization and contributes to tumorigenesis. [Cancer Res 2008;68(11):4258–68]

Introduction

In recent years, several groups of proteins involved in cell/cell adhesion complexes were shown to play an important role in intracellular cell signaling (1, 2). This is notably the case for tight junction (TJ) proteins, which have been identified as regulators of cell proliferation in kidney and intestinal cells (3, 4). Among these proteins is the large family of claudin isoforms, which have been described as essential modulators of paracellular permeability, and whose role on intracellular signaling (5, 6) and on the expression of the cancer cell phenotype (7) is slowly emerging. Indeed, both overexpression and down-regulation of selective claudin isoforms have been identified in various types of cancers, resulting in a disruption of TJ strands and an increase in epithelial permeability (8, 9). Several processes have been suggested to play a role in the promotion of tumor development upon claudin/TJ disruption, including a facilitation of growth factor leakage from the lumen (8), a promotion of proliferation (2, 7), a role in the loss of polarity (9) and the appearance of invasive behavior (10) in epithelial cells.

In this context, it is important to precisely identify the regulators of claudin expression and localization in specific areas, and to determine the contribution of each isoform to cell and organ physiology. Alteration of the expression and/or localization of several claudin isoforms has been reported in the colon (11–14). The best described of these isoforms is claudin-1, which has been identified as a target of the Wnt/β-catenin pathway (14). Overexpression of claudin-1 in human tumors promotes cell invasion (10, 15) and is a strong prognostic indicator in stage II colon cancer (16). Another claudin isoform, claudin-7, was also recently shown to be overexpressed in human colon cancer samples (13, 17). Claudin-7 displays a peculiar expression profile in epithelial cells of several organs, largely outside the TJ area and along the basolateral membrane (18–20). Factors regulating claudin-7 expression are largely unknown, although it was shown to be under the control of androgens in the prostate (21). In addition, hypermethylation of the claudin-7 gene promoter was reported as being responsible for the down-regulation of this protein in a panel of breast cancer cell lines (22).

To date, the precise biological role of claudin-7 remains elusive. Experimental modulation of its expression regulates the paracellular leakage of Cl^- and Na^+ in kidney cells (23), and a potential role of claudin-7 in cell differentiation has been suggested in the prostate, where claudin-7 regulates prostate-specific antigen expression (21). More recently, claudin-7 up-regulation in esophageal carcinoma cells was shown to increase E-cadherin expression and adhesive properties, and to reduce their invasive potential (24). Our current understanding of the biological roles played by claudin-7 is further clouded by the fact that, depending on the organ, both its down-regulation (in the breast and esophagus;

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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doi:10.1158/0008-5472.CAN-07-5805

refs. 22, 24) or its overexpression (e.g., in the stomach and kidney; refs. 25, 26) have been strongly associated with the tumorigenesis process.

Consequently, the objectives of the present work were to identify factors regulating claudin-7 expression in the colon, and to analyze the consequences of its overexpression on the polarization, proliferation, and tumorigenicity of colorectal cancer cells.

Materials and Methods

DNA constructs. The antisense SOX-9 expression construct and the GFP-Cter-E-cadherin construct were described previously (27). pEF/myc/cyto vectors (Invitrogen) and ΔN87 β-catenin with COOH-terminal myc epitope ligated into vector at *Nco*I-*Pst*I sites (28) were generously provided by Dr. Y. Yeom (Functional Genomics Research Center, Daejeon, Republic of Korea). The CLDN-7 promoter luciferase construct (29) was a generous gift from Dr. J. Toguchida (Institute for Frontier Medical Sciences, Kyoto, Japan).

Cell culture. Two-dimensional cultures of SW480 and HT29-Cl.16E/Sox-9 cells were performed exactly as described in ref. (30) and ref. (27), respectively, except that mRNA and protein expression in the latter were analyzed 6 days after doxycycline induction.

For three-dimensional cultures, 1×10^5 HT29-Cl.16E cells were carefully mixed in a total volume of 300 μL of Matrigel basement membrane matrix (Becton Dickinson) and seeded into the upper chamber of a 24-well cell culture insert (Becton Dickinson). The cell/Matrigel mixture was incubated at 37°C, 5% CO_2 for 30 min before cell culture medium was added to the bottom chamber and on top of the Matrigel. The cultures were treated with 1 μg/mL of doxycycline where appropriate, and the medium was renewed every third day for 2 weeks. Matrigel cultures were then snap-frozen in embedding medium (Tissue-Tek, Sakura) and stored at −80°C. Twenty-micrometer-thick sections were cut at −20°C using a cryomicrotome (Microm) and mounted on SuperFrost or Polylysine microscope slides for immunofluorescence experiments. To analyze proteins from three-dimensional culture cells, cell/Matrigel were incubated with trypsin for 30 min at 37°C and then lysed in 1× radioimmunoprecipitation assay (RIPA) buffer. We resolved lysates by SDS-PAGE, transferred them to nylon membranes and subjected them to immunoblotting.

Transfection and reporter gene assays. DN87 β-catenin, antisense Sox-9, and the CLDN-7 promoter luciferase (−721; 0.5 μg of each DNA/well) were cotransfected into SW480 cells cultured on 24-well dishes. Transfections were carried out in triplicate using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. Forty-eight hours after transfection, firefly luciferase and *Renilla* luciferase activities were measured using the Dual Luciferase Reporter Assay System (Promega) according to the instructions of the manufacturer. Tcf-4 transcriptional activity was quantified as described in ref. (30). The activity of SOXs transcription factors was quantified using "SOX luciferase" and control "SAC luciferase" reporter constructs (a gift from Prof. H. Clevers, Hubrecht Laboratory, Utrecht, the Netherlands), as previously described (31).

Small interfering RNA transfections. Small interfering RNAs (siRNA) for the CLDN-7 gene consisted on a cocktail of three siRNAs, designed by Ambion's silencer and purchased from Eurogentec (sequences are supplied in Supplemental Table S1). SW480 cells were seeded into six-well plates at 2×10^5 cells/well and transfected the following day with 120 nmol/L siRNA cocktail using Lipofectamine 2000 (Invitrogen). After transfection (72–96 h), cells were harvested and 1× RIPA protein lysates were prepared to assess the knockdown effect of siRNAs on the expression of CLDN-7.

CLDN-7 lentiviral vector and infection. The pHR′-CLDN-7 lentiviral transduction plasmid (21) was a generous gift from Dr. S. Pang (University of California at Los Angeles Dental Institute and Jonsson Comprehensive Cancer Center, Los Angeles, CA) and has been described previously. This plasmid was used to generate a corresponding lentiviral vector by a cotransfection method. Infection of 2×10^5 HT29-Cl.16E cells has been performed in two-dimensional cultures plated into six-well tissue culture plates 24 h prior to infection. Five micrograms of viral vector (multiplicity of infection = 5) were added to each well in the presence of 4 μg/mL polybrene (Sigma) to facilitate infection 24 h postinfection, the cells were washed and fresh medium was added. The cells were then counted and used to infect three-dimensional cultures in Matrigel.

Western blot. Cells were lysed in 1× RIPA buffer, and 20 to 50 μg of proteins were loaded per lane on 7% to 12% SDS-PAGE gels, then transferred onto nitrocellulose membranes as previously described (32). Primary antibody dilutions are provided in Supplemental Table S1 online. Detection was performed using the ECL plus system (Amersham). Optimal exposure times of membranes were used and protein expression was quantified using NIH Image 1.62, and adjusted for background noise and protein loading.

Tissue samples. Specimens of colon tumors and matching healthy epithelium, as well as paraffin-embedded sections from 12 patients were obtained from the pathologist after resection according to French government regulations and with approval of the ethical committee (Nîmes Hospital).

The intestine of the Sox-$9^{\Delta/\Delta}$ mice (30) was dissected out, rinsed in PBS, then separated into four segments corresponding to the duodenum, jejunum, ileum, and colon. The lumen was cleaned by gentle PBS flushing, and each segment was rolled and placed into a paraffin-embedding cassette, fixed for 4 h at room temperature in 4% paraformaldehyde, rinsed in PBS, and paraffin-embedded using standard procedures (31).

Immunofluorescence and confocal microscopy. Cells were grown on glass coverslips in 12-well plates until the confluent stage, and immunofluorescent staining was performed as described in ref. (30). Samples were mounted in mowiol (Aldrich) and examined using a Bio-Rad MRC 1024 confocal laser scanning setup [Optiphot2 microscope, with a Plan Apo 40×/0.75 oil-immersion objective lens (Nikon)]. Digitized fluorescent images excited by a crypton/argon laser were acquired (1,024 × 1,024 pixel frame memory). Confocal images were mounted as figures using Adobe Photoshop 7.0 software (Adobe Systems Incorporated).

Immunohistochemistry. Sections were deparaffinized using standard procedures. Antigen retrieval, immunostaining, counterstaining and sample observation were performed as described in ref. (30).

Reverse transcription-quantitative PCR. Extraction of total RNA with the RNeasy kit (Qiagen), reverse transcription, and quantitative PCR amplification have been described previously (30). Primer sequences are supplied in Supplemental Table S1.

Growth of HT29 xenografts in *nude* mice. *In vivo* experiments were performed in compliance with the French guidelines for experimental animal studies (Direction des Services Vétérinaires, Ministère de l'Agriculture, agreement no. B 34-172-27) and fulfill the U.K. Coordinating Committee on Cancer Research guidelines for the welfare of animals in experimental neoplasia. BALB/c *nu/nu* (*nude*) mice were injected s.c. with 10^7 of HT29-Cl.16E/Sox-9 cells infected with the control virus in one flank and with the same amount of cells infected with the claudin-7–expressing virus on the opposite side. All mice were then provided with doxycycline in the drinking water. Tumor growth was measured 6 weeks after injection [estimated tumor volume = (length × width × thickness) / 2].

Results

Increasing expression gradient of claudin-7 along the crypt/villus axis of colonic crypts and overexpression in colorectal tumor samples and cell lines. We first analyzed the expression pattern of claudin-7 in the human colonic epithelium using immunohistochemistry and immunofluorescent staining. Claudin-7 was expressed throughout the crypts, but both the intensity and nature of staining varied from the basal compartment to the surface epithelium. Differentiated cells at the surface displayed a very strong claudin-7 expression (Fig. 1*A*), whereas claudin-7 staining was much weaker at the crypt base (Fig. 1*A*). In addition, variations of claudin-7 localization were also detected within individual cells, from a basolateral membrane distribution in differentiated cells to a more restricted distribution at the basal membrane and the TJ at the base of colonic crypts (Fig. 1*B*). Similar

results were obtained using a monoclonal and a polyclonal antibody against claudin-7. These results indicate that claudin-7 expression is weaker in the proliferating compartment of colonic crypts, in which the Wnt/Tcf-4 transcriptional program is activated, as confirmed by CD44 and Sox-9 staining (Fig. 1*A*). In contrast, we found that claudin-7 was strongly overexpressed in colorectal carcinoma (CRC) samples, even in areas with very high Tcf-4 activity (Fig. 1*C*).

In addition, the expression of claudin-7 and Tcf-4 target genes was also analyzed in a panel of cell lines. All CRC cell lines tested (Caco2, SW480, DLD-1, HCT116, HT-29) expressed high levels of claudin-7, whereas expression of c-Myc and cyclin D1 was found to be significant but variable, reflecting the previously described (33) heterogeneous amplitude of Tcf-4 activity in these APC- or β-catenin–mutated cells. In nontumoral cell lines, claudin-7 expression was elevated in the highly differentiated Madin-Darby canine kidney (MDCK) cells, which displayed low Tcf-4 target gene expression, whereas claudin-7 expression was very low in the poorly differentiated mouse colonic cell line YAMC, in which Tcf-4 activity is significantly higher (Fig. 1*D*).

These results support the existence of an opposite expression gradient between claudin-7 and Tcf-4 target genes in healthy human colonic crypts and in nontumoral cell lines, and suggest that this opposite gradient is partially lost in colorectal tumor samples and cell lines.

Claudin-7 expression is negatively regulated by the Wnt/Tcf-4 pathway. We then proceeded to determine whether transient modulation of Tcf-4 activity would be sufficient to regulate the expression of claudin-7. To this effect, SW480 cells were used in view of their high transfection capacity, and Tcf-4 activity was stimulated or inhibited by expressing a nondegradable β-catenin mutant (DN87-β-catenin, ref. 28) or the soluble COOH-terminal domain of E-cadherin (27), respectively. Overexpression of the DN87-β-catenin mutant was capable of further stimulating Tcf-4 activity in SW480 cells, as confirmed by TOP/FOP Tcf-4 reporter assay (Fig. 2*A*) and by quantification of Tcf-4 target gene expression, including claudin-1 and claudin-2 (Fig. 2*B* and *C, left*). It also induced a mild but significant decrease of claudin-7 expression (Fig. 2*B* and *C*). On the other hand, experimental inhibition of the Wnt/Tcf-4 pathway increased claudin-7 expression while leaving the expression of claudin-4 unaffected and repressing that of the recognized Tcf-4 targets c-Myc, cyclin D1, claudin-1, and claudin-2 (Fig. 2*C*). Similar experiments were then performed in the nontumoral cell line YAMC. We found that the modulation of Tcf-4

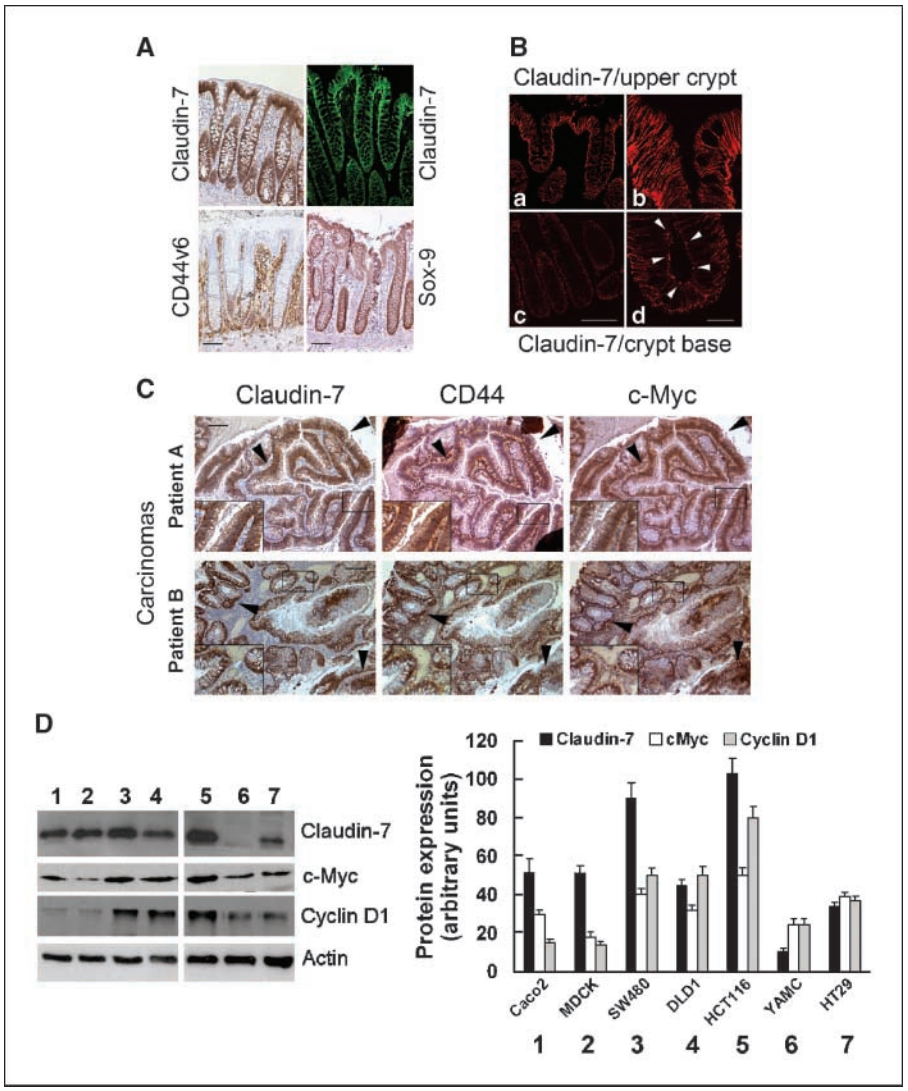

Figure 1. Expression pattern of claudin-7 in human colonic crypts and colorectal tumors. *A,* representative images of claudin-7, CD44v6, and Sox-9 detection by immunohistochemistry or immunofluorescent staining in human colonic tissue sections. *Bar,* 100 μm. *B,* differential distribution of claudin-7 along human colonic crypts was detected using immunofluorescent staining as above. Low (*a* and *c*) and high (*b* and *d*) magnification representative images are provided of the surface epithelium (*a* and *b*) or the crypt base (*c* and *d*). *Bars,* 100 μm (*a* and *c*) or 20 μm (*b* and *d*). *White arrowheads,* claudin-7–enriched TJs (*d*). *C,* representative images of claudin-7, c-Myc, and CD44 immunohistochemistry in serial sections from paraffin-embedded human colorectal adenocarcinoma samples. *Bar,* 100 μm. *Black arrowheads,* areas where all three proteins are strongly expressed. *Insets,* boxed areas in the main pictures (original magnification, ×3). *D,* Western blot analysis (*left*) and quantification (*right*) of claudin-7, c-Myc, cyclin D1, and Sox-9 expression in Caco2 (*1*), MDCK (*2*), SW480 (*3*), DLD-1 (*4*), HCT116 (*5*), YAMC (*6*), and HT-29Cl.16E (*7*) cells. Actin expression is shown as a loading control. *Columns,* mean from three independent experiments; *bars,* SE.

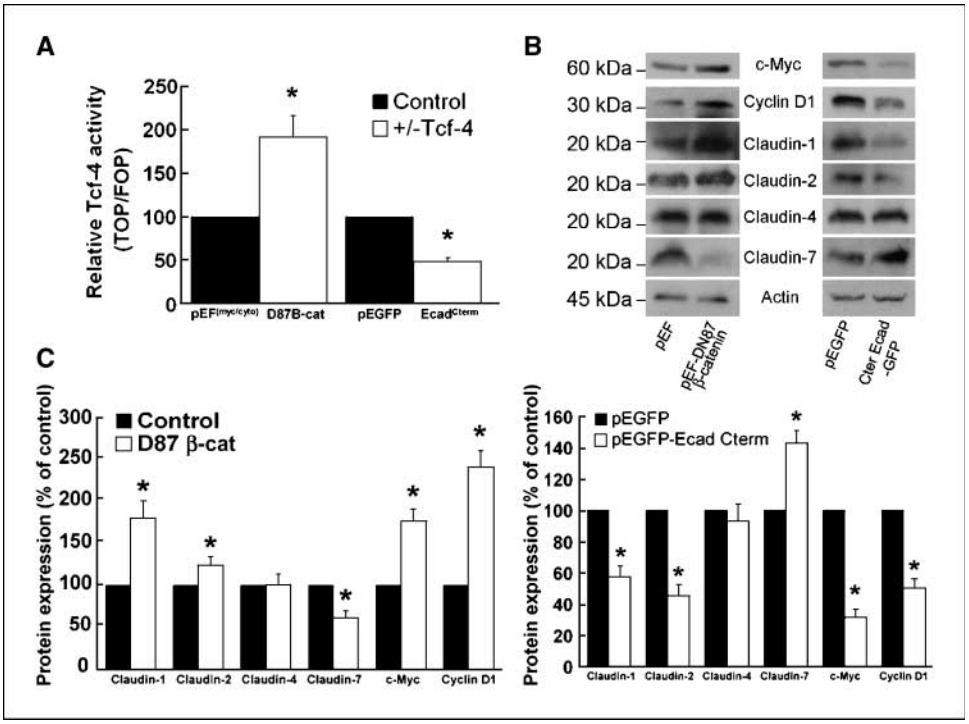

Figure 2. Claudin-7 expression is negatively regulated by the Wnt/Tcf-4 pathway. *A,* SW480 cells were transiently transfected with the pEF vector alone or containing the ΔN87-β-catenin mutant, or with the pEGFP vector alone or containing the Cterm domain of E-cadherin, and the transcriptional activity of Tcf-4 was analyzed using the TOP/FOP assay (*columns,* mean; *bars,* SE; $n = 3$). *B,* SW480 cells were transfected as in *A*, and the expression of c-Myc, cyclin D1, claudin-1, claudin-2, claudin-4, and claudin-7 were analyzed using Western blotting. Actin expression is shown as a loading control. *C,* histograms summarizing the quantification of Western blot results in *B*, from three independent experiments. *, $P < 0.05$ compared with controls (Student's *t* test).

activity and target gene expression was weaker in these cells when compared with SW480 cells (Supplemental Fig. S1), maybe because nontumoral cells have a greater ability to endogenously balance their Tcf-4 activity than tumor cells. Alternatively, this result could be due to a smaller transfection efficiency in YAMC cells, or to a lesser efficiency of the DN87-β-catenin and soluble COOH-terminal E-cadherin constructs on mouse Tcf-4. In spite of this, modulation of claudin-7 expression in these cells (Supplemental Fig. S1) was at least as strong as what was detected in SW480 cells.

Taken together, these results indicate that activation of the Tcf-4 transcriptional program represses claudin-7 expression in colonic cells, and suggests that the efficiency of this repression is decreased, if not abolished, in CRC cells.

Sox-9 mediates Wnt/Tcf-4 signals on claudin-7 expression. *In silico* analysis indicated that no Tcf-4 binding sites are present on the claudin-7 promoter, suggesting that the repressive effect identified above might be indirect. Our attention turned towards the high mobility group (HMG)–box transcription factor Sox-9 as a potential intermediate between Tcf-4 and claudin-7 repression because (*a*) it was previously identified as a mediator for some of the Tcf-4 transcriptional programs (27, 30), notably the repression of differentiation-associated genes (27), and (*b*) preliminary data obtained after analysis of microarray-based gene profiling in a previously described model of inducible Sox-9 overexpression in the differentiated colorectal cancer cell line HT-29Cl.16E (27, 31), suggested that Sox-9 could modulate claudin expression.

Similar to what was found in human CRC samples (27), we first found that high levels of Sox-9 expression were detectable in HT-29Cl.16E and in SW480 CRC cell lines whereas, in contrast, Sox-9 expression was very low in DU145 prostate carcinoma cells which were used as controls (Supplemental Fig. S2). Sox activity was quantified using the previously described SOX/SAC luciferase assay (31), demonstrating that Sox activity was weak in SW480 and HT-29 cells when compared with DU145 cells (Supplemental Fig. S2). Using the previously described inducible HT-29Cl.16E/Sox-9 cell line (31), we were then able to show that Sox-9 induction repressed the expression of claudin-7, while increasing the expression of the crypt base–specific isoform claudin-2 and slightly decreasing that of claudin-1 (Fig. 3*A*). This result recapitulates the effects of Tcf-4 activation and is consistent with the expression pattern of Sox-9 and of the corresponding claudin isoforms within the colonic epithelium (cf. Fig. 1; ref. 34), except in the case of claudin-1, which is evenly expressed throughout colonic crypts. *In vivo*, we detected a very strong overexpression of claudin-7 throughout the colonic epithelium of recently described Sox-9–deficient mice (30), confirming the existence of a "constitutive" repression of claudin-7 by Sox-9 in the colon (Fig. 3*B*). In contrast, claudin-2 expression seemed to be normal in these animals, indicating that Sox-9 is not essential for mediating the effects of Tcf-4 on claudin-2 expression in the colonic crypts. Repression of a (−721/+1) claudin-7 promoter fragment coupled to a luciferase reporter was induced after Sox-9 induction in HT-29Cl.16E cells (Fig. 3*C, top*), although significant induction of this reporter gene was detected after the expression of a Sox-9 antisense construct (Fig. 3*C, bottom*). Finally, cotransfection experiments showed that the down-regulation of claudin-7 expression induced by hyperactivation of the Tcf-4 program with an activated form of β-catenin was reversed by the presence of an antisense form of Sox-9 (Fig. 3*D*), indicating that Sox-9 was essential to mediate the Tcf-4–driven repression of claudin-7 in these cells. Expression of the antisense Sox-9 construct in control cells was also found to enhance claudin-7 expression, suggesting that a degree of basal Sox-9 activity must exist in these cells (Fig. 3*D*).

Because Sox-9 has not been described as a direct transcriptional repressor, and because Sox-9–induced repression of cdx2 and Muc2 in the intestine was shown to necessitate the involvement of at least one intermediate protein (27), it is likely that the same holds true for the regulation of claudin-7 expression. In view of the

overrepresentation of Snail and Snail2/Slug binding sites in the claudin-7 promoter sequence (35), and because Snail2 was recently described as a Sox-9 target in early neural crest development (36), we quantified the expression of these two transcriptional repressors in HT-29Cl.16E cells before and after Sox-9 induction or Sox inhibition using a previously described dominant-negative Sox-9 construct, but were unable to detect any changes in their mRNA levels (Supplemental Fig. S3 online).

Taken together, these results indicate that Sox-9 is capable of repressing claudin-7 activity in mouse and human colon, and is an essential intermediate of Tcf-4's effect on this protein. They also suggest that decreased Sox-9 activity could be responsible for the dysfunctional claudin-7 repression by Tcf-4 in CRC cells.

Decreased claudin-7 expression is concomitant with an increased cell polarization in Sox-9–overexpressing cells. Using immunofluorescence, we then comparatively analyzed the distribution of claudin-7 and claudin-2 along the *XY*-axis and *XZ*-axis in HT-29Cl.16E/Sox-9 cells grown in two-dimensional cultures. Claudin-7 was found all along the lateral membrane of noninduced HT-29Cl.16E/Sox-9 cells, whereas claudin-2 was found exclusively at the TJ (Fig. 4*A*). In contrast, residual claudin-7 detected in Sox-9–overexpressing cells was mostly colocalized with claudin-2 at the TJ (Fig. 4*A*), similar to what was detected in cells located at the crypt base within the colon (Fig. 1). To determine whether claudin-7 modulation was concomitant with modifications of cell polarization, we used a three-dimensional culture system within Matrigel, in which HT-29Cl.16E cells grow as clusters without any detectable organization or polarization (Fig. 4*B*). The induction of Sox-9 under these conditions led to a drastic change in morphology, with the appearance of a lumen in >80% of spheroids and an increase in cell polarization, characterized by the concentration of ezrin and protein kinase C-ζ at the apical side of cell membranes, bordering the lumens (Fig. 4*B*). Protein expression of the polarity markers ezrin and villin was slightly reduced after Sox-9 induction, whereas both the expression of the proliferation marker PCNA (proliferating cell nuclear antigen) and the detection

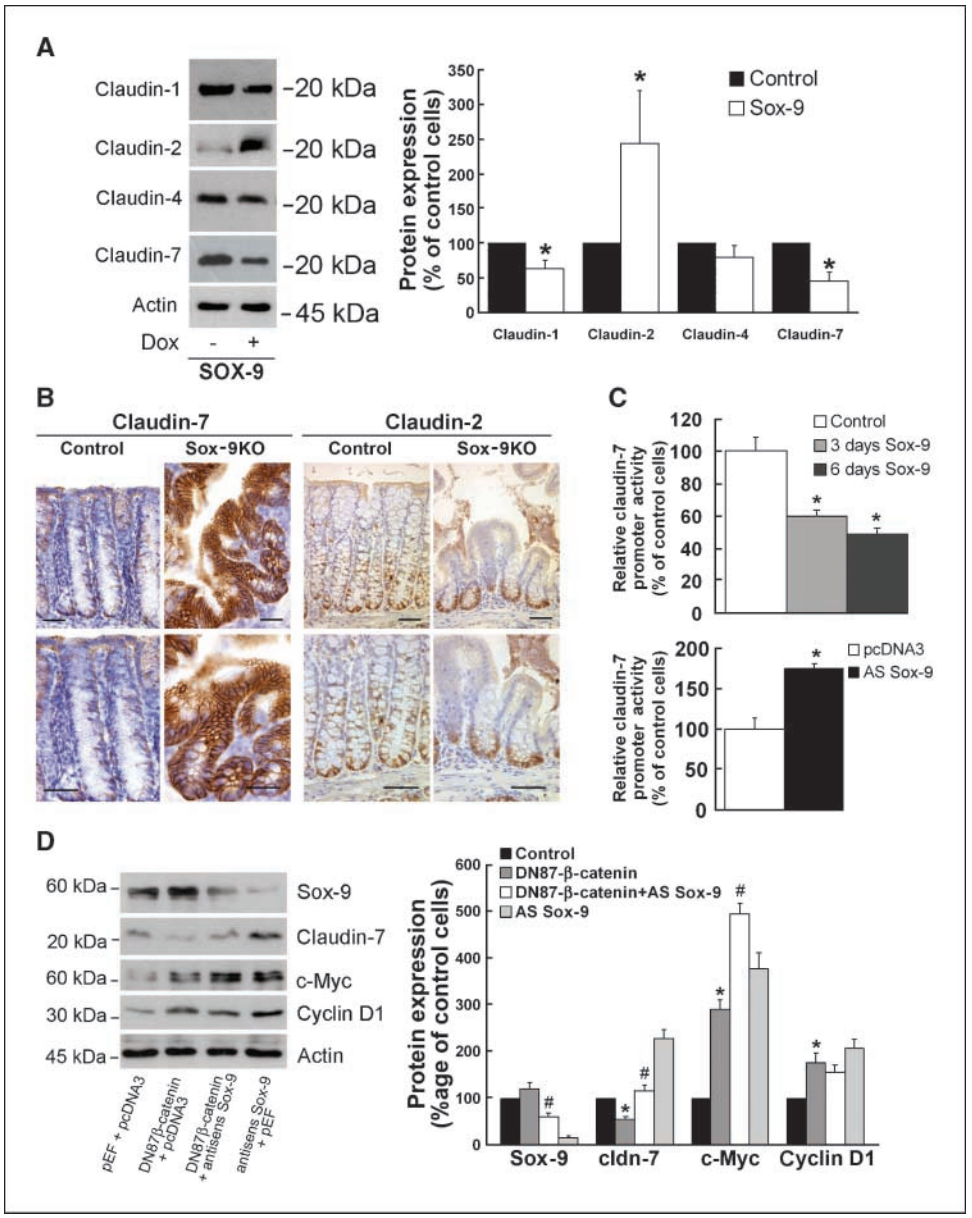

Figure 3. Sox-9 is an essential mediator of Wnt-induced repression of claudin-7. *A,* expression of claudins 1, 2, 4, and 7 was quantified using Western blotting in HT-29Cl.16E/Sox-9 cells (*left*) induced or not with doxycyclin as indicated under Materials and Methods. *Columns,* mean from three independent experiments; *bars,* SE (*right*). *, $P < 0.05$ compared with control cells (Student's *t* test). *B,* tissue sections from the colon of control and Sox-9–invalidated mice (30) were stained with antibodies against claudin-2 or claudin-7 and counterstained with hematoxylin. *Bars,* 100 μm. *C,* luciferase reporter assay reflecting the activity of the claudin-7 (−721/+427) promoter fragment after Sox-9 induction in HT-29Cl.16E cells (*top*) or after transfection of a control or an antisense Sox-9 construct in SW480 cells (*bottom*). *, $P < 0.05$ compared with control cells (Student's *t* test). *D,* SW480 cells were transiently transfected with the pEF vector alone or containing the ΔN87-β-catenin mutant, as well as with pcDNA3 alone or carrying and antisense Sox-9 construct as indicated. Cells were lysed 48 h after transfection and the expression of Sox-9, claudin-7, c-Myc, and cyclin D1 was analyzed using Western blotting. *Right,* quantification of proteins from three independent experiments was performed after normalizing for protein loading, using actin as a standard. *, $P < 0.05$ compared with control cells; #, $P < 0.05$ compared with ΔN87-β-catenin–expressing cells (Student's *t* test).

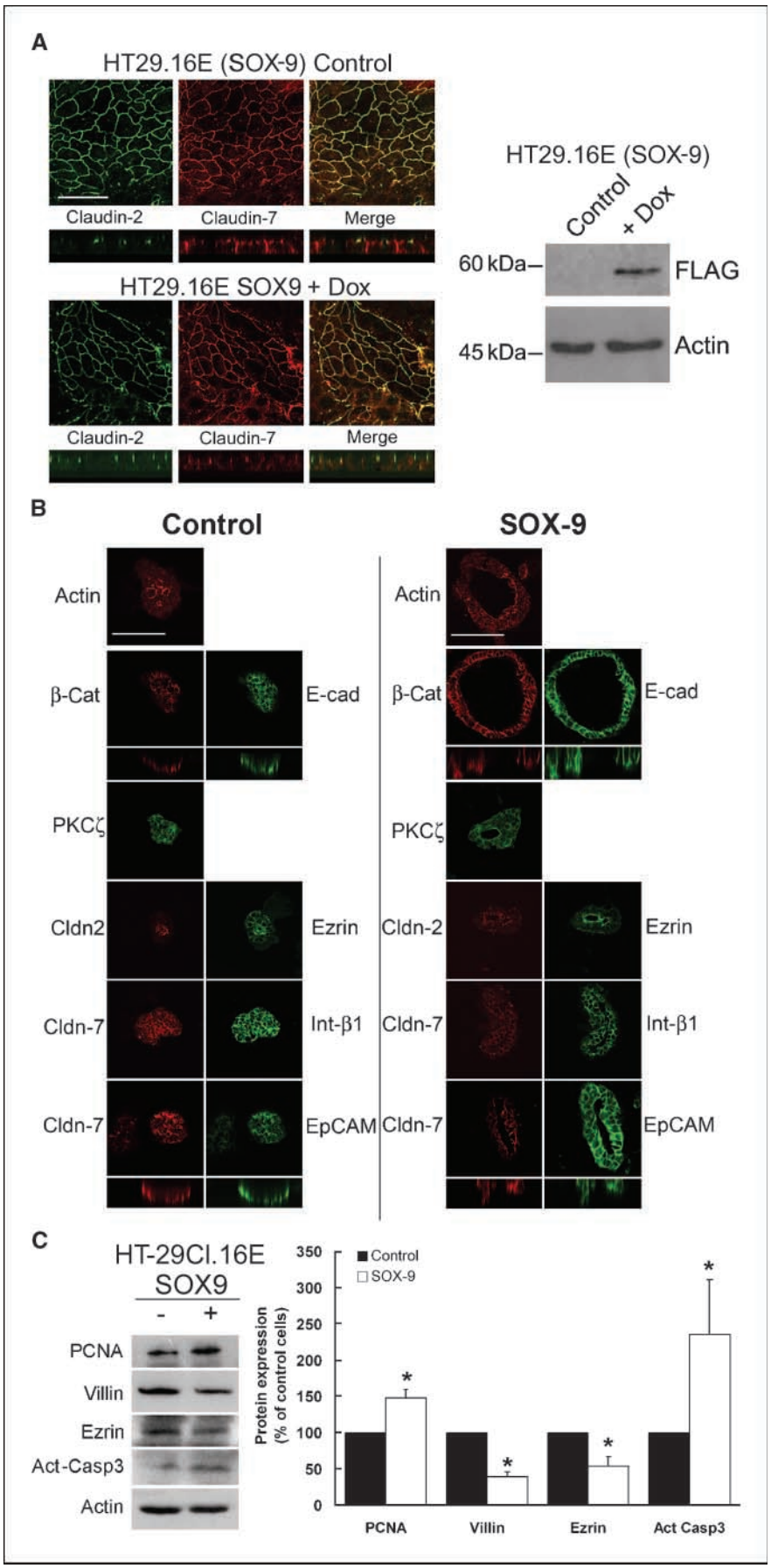

Figure 4. Increased cell polarization and TJ localization of residual claudin-7 upon Sox-9 induction in HT-29.Cl16E CRC cells. *A,* HT-29Cl.16E/Sox-9 cells were grown to confluence and Sox-9 expression was induced (+ *Dox*) or not (*control*) via doxycyclin treatment as described under Materials and Methods. *Left,* cells were then fixed and stained using claudin-2 and claudin-7 antibodies, followed by observation along the *XY*-axis and *XZ*-axis using a Bio-Rad 1024 confocal microscope. *Bar,* 50 μm. Note that the intensity of claudin-7 staining after Sox-9 induction (*HT29.16E SOX9 + Dox*) was evenly enhanced across *XY* and *XZ* images to allow better detection of residual claudin-7 localization. *Right,* cells were lysed and expression of FLAG-tagged exogenous Sox-9 was confirmed after doxycyclin induction using Western blotting, as previously described (27, 31). Actin was used as a loading control. *B,* HT-29Cl.16E/Sox-9 cells were grown in a three-dimensional environment, treated or not with doxycyclin, and fixed as described. Sections were stained using the indicated antibodies and observed along the *XY*-axis, and sometimes *XZ*-axis, using a Bio-Rad 1024 confocal microscope. *Bars,* 50 μm. *C,* cells were grown as above, then lysed, and the expression of PCNA, villin, ezrin and activated caspase 3 was quantified using Western blotting (*left*). Histogram summarizing the protein quantification from three independent experiments (*right*). *, $P < 0.05$ compared with control cells (Student's *t* test).

of the activated form of caspase 3 were significantly increased under these conditions (Fig. 4*C*), reflecting an increased cell turnover in Sox-9–overexpressing cells. We were unable to perform similar experiments on SW480 or YAMC cells, which in our hands, never formed spheres under three-dimensional culture conditions in Matrigel or in suspension.

These results suggest that, in response to Sox-9 induction, the down-regulation and TJ focusing of claudin-7 is concomitant with an overall increase in the polarization and reorganization of differentiated CRC cells into organoid-like structures.

Claudin-7 overexpression disrupts cell polarity and increases Tcf-4 activity, proliferation, and tumorigenicity of CRC cells. To better understand the potential biological consequences of claudin-7 overexpression for tumorigenesis, we infected Sox-9–overexpressing HT-29Cl.16E cells with a lentivirus encoding claudin-7 (21), thus restoring the concomitant expression of Tcf-4 target genes and claudin-7 detected in human colorectal tumors (ref. 13; Fig. 1). Claudin-7–overexpressing cells grew significantly faster in two-dimensional cultures when compared with cells infected with a control virus (Fig. 5*A*). In three-dimensional Matrigel cultures, the organization and polarity of spheres overexpressing claudin-7 were completely disrupted, with most spheres found to be devoid of a lumen and polarity markers displaying weak and diffuse staining (Fig. 5*B*). In addition, staining for the proliferation marker PCNA, as well as for the Tcf-4 targets c-Myc and cyclin D1, were strongly enhanced after infection with the claudin-7 viral construct. The overall pattern of apoptosis was also found to differ after claudin-7 overexpression. Apoptotic cells were always detected towards the lumen of HT-29Cl.16E/Sox-9 spheres, whereas apoptotic activity was slightly higher and located towards the periphery of claudin-7–overexpressing spheroids (Fig. 5*B*). The increased expression of Tcf-4 targets following claudin-7 overexpression was confirmed using Western blotting from three-dimensional sphere lysates (Fig. 5*C*), or the TOP/FOP Tcf-4 activity reporter assay in two-dimensional cultures (Supplemental Fig. S4 online). Conversely, siRNA-mediated transient down-regulation of claudin-7 expression in SW480 colorectal cancer cells decreased the expression of c-Myc and cyclin D1 (Fig. 5*D*), whereas claudin-4 expression was unaffected. A mild inhibition of claudin-1 expression was also detected in cells transfected with the claudin-7 siRNA, most likely because the CLDN1 gene is also a Tcf-4 target.

Finally, the consequences of claudin-7 overexpression were then analyzed on the anchorage-independent growth *in vitro* and on the tumor-forming ability of colorectal cancer cells *in vivo*. *In vitro*, we found that the size of claudin-7–overexpressing HT-29Cl.16E/Sox-9 cell spheroids in three-dimensional Matrigel cultures was much larger than that of uninfected cells or cells infected with a control virus (Fig. 6*A*). *In vivo*, HT-29Cl.16E/Sox-9 cells expressing the control or claudin-7–expressing virus were injected s.c. in contralateral flanks of BALB/c *nude* mice, which were provided with doxycyclin in their drinking water. Large tumor xenografts were measured 40 days after injection from claudin-7–overexpressing cells only (five of six mice), whereas no tumors were detectable from control cells during this time frame (zero of six mice; Fig. 6*B*), demonstrating that claudin-7 overexpression promotes the tumor-forming ability of these cells *in vivo*.

Taken together, these results strongly suggest that claudin-7 overexpression induces a loss of polarization, enhances β-catenin/Tcf-4 activity and proliferation, and promotes the tumorigenicity of colorectal cancer cells.

Discussion

The first important finding of the present work is that the Wnt/Tcf-4 pathway negatively regulates the expression of claudin-7, resulting in the presence of an increasing gradient of expression for this protein from the bottom of human colonic crypts towards the differentiated surface epithelium. Although the presence of claudin-7 in the colonic epithelium has been previously reported (13, 17), our work provides, to our knowledge, the first detailed description of the expression pattern of claudin-7 within the human colon. Detection of this gradient was confirmed by using both immunofluorescent and immunohistochemical staining using two independent claudin-7 antibodies. This expression profile is opposite to that of claudin-2 (34), which was identified as a positively regulated target of Tcf-4 in mammary epithelial cells (37). In view of the differential involvement of claudin isoforms in paracellular permeability modulation (23, 38), this result suggests that the permeability to ions could vary in different areas within individual colonic crypts. Although this gradient of expression was not detected for claudin-7 in the mouse colonic epithelium, it exactly recapitulates that identified for claudin-13 (39), indicating that different claudin isoforms could play a similar role in human and mouse. In addition, because claudins and other TJ proteins have been recently identified as signaling effectors (2, 7, 9), their differential expression in the proliferating and differentiated compartment of colonic crypts could also imply that they do have a role to play in the differentiation process of intestinal cells, as recently suggested (40, 41). In this respect, it is noteworthy that claudin-1 seems to be evenly distributed from the crypt base to the surface epithelium (14), although it was shown to be a Wnt/Tcf-4 target and to play a role in tumorigenesis of the colon (10, 14).

In addition, our data shows that the HMG-box transcription factor Sox-9 is an essential mediator of the Tcf-4–driven down-regulation of claudin-7. This result is in agreement with the preferred distribution of Sox-9 at the base of colonic crypts (27), where we found claudin-7 expression to be the lowest. Sox-9 is a HMG-box transcription factor involved in the regulation of differentiation processes in several organs (42–44), and was recently shown to repress differentiation-associated genes such as cdx2 and Muc2 in the intestine (27). Recent results by our laboratory and that of others also showed that tissue-specific deletion of this transcription factor induced severe morphologic alteration in the small intestine and colon (30, 45). The present data, which shows that high Sox-9 activity is essential for the polarization of HT-29 cells in a three-dimensional context, corroborates the hypothesis that Sox-9 plays an important role in the specification of cell and tissue architecture within the intestine. Interestingly, claudin-7 was almost exclusively found towards the TJ area after Sox-9 induction, similar to what is found *in vivo* at the colonic crypt base, where Sox-9 expression is high. In contrast, exogenous claudin-7, overexpressed using a viral construct, was localized along the whole basolateral membrane in three-dimensional culture, resulting in a loss of general organization and polarity of HT-29Cl.16E spheroids. The molecular mechanism underlying this role still needs further clarification, although previously described roles of claudin-7 in the clustering of EpCAM and CD44 into tetraspanin-enriched membrane microdomains (13) and in the modification of cell-matrix interactions (18) could be involved. In contrast with these results and ours, up-regulation of CLDN7 in esophageal carcinoma cells was reported to increase adhesive properties and to reduce invasion, mostly through a significant increase of E-cadherin expression

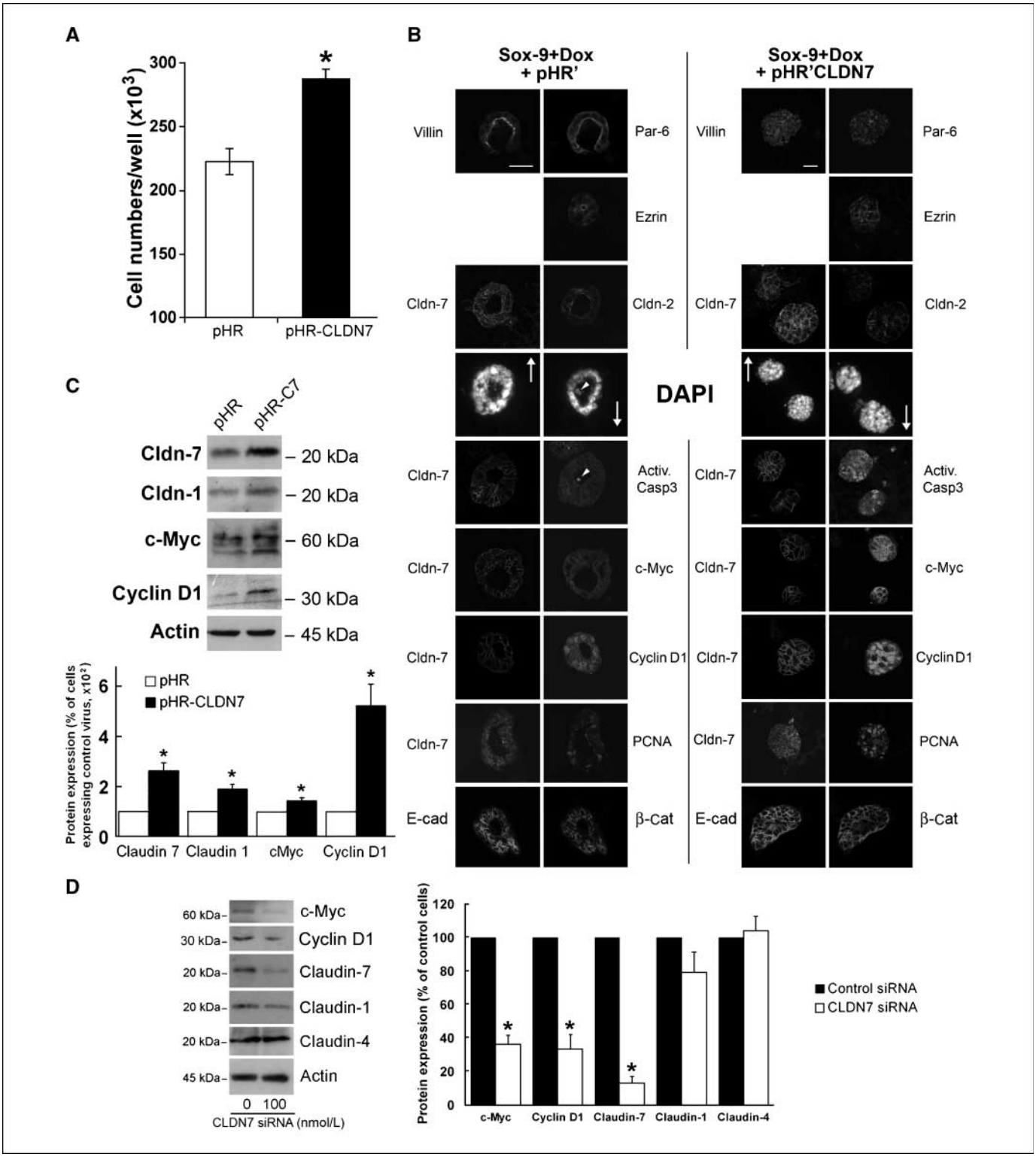

Figure 5. Claudin-7 overexpression in CRC cells enhances proliferation and Tcf-4 activity and disrupts cell polarization. *A,* HT-29Cl.16E/Sox-9 cells were infected with control or claudin-7–expressing viral constructs, grown in two-dimensions for 72 h, resuspended using Trypsin/EDTA and counted using a cytometer. Results summarize the counts from three independent experiments in triplicate wells. *B,* sections from fixed HT-29Cl.16E/Sox-9 spheres grown in three-dimensions were prepared as described and stained for cell polarization markers (villin, Par6, ezrin), claudin-2 and claudin-7, E-cadherin, β-catenin, Wnt target genes (c-Myc, cyclin D1), as well as proliferation and apoptosis markers. *White arrows,* correspondence between 4′,6-diamidino-2-phenylindole and related IF staining. *White arrowheads,* positive staining for activated caspase 3. *Bars,* 50 μm. *C,* representative Western blot showing the expression of claudins and Wnt target genes in three-dimensional HT-29Cl.16E/Sox-9 spheres after infection with control or claudin-7-expressing viral constructs (*top*). Quantification from three similar experiments (*bottom*). *D,* expression of c-Myc, actin and claudin-1, -4, and -7 was analyzed using Western blotting in lysates from HT-29Cl.16E/Sox-9 cells grown in two dimensions and transfected with the indicated concentration of CLDN7 siRNA. The histogram summarizes the quantification from three independent experiments (*right*); *, $P < 0.05$ compared with pHR-infected cells (in *A* and *C*), and with control cells (*D*). Student's *t* test.

(24). However, we did not measure any modifications of E-cadherin expression or localization upon claudin-7 overexpression in HT-29Cl.16E CRC cells, whereas the strong morphologic alterations detected in the present work were not reported by Lioni et al. (24), suggesting that the biological role of claudin-7 could vary significantly depending on the tissue.

The present data, as well as recent results by Kuhn et al. (13) indicate that high claudin-7 expression is no longer exclusive from,

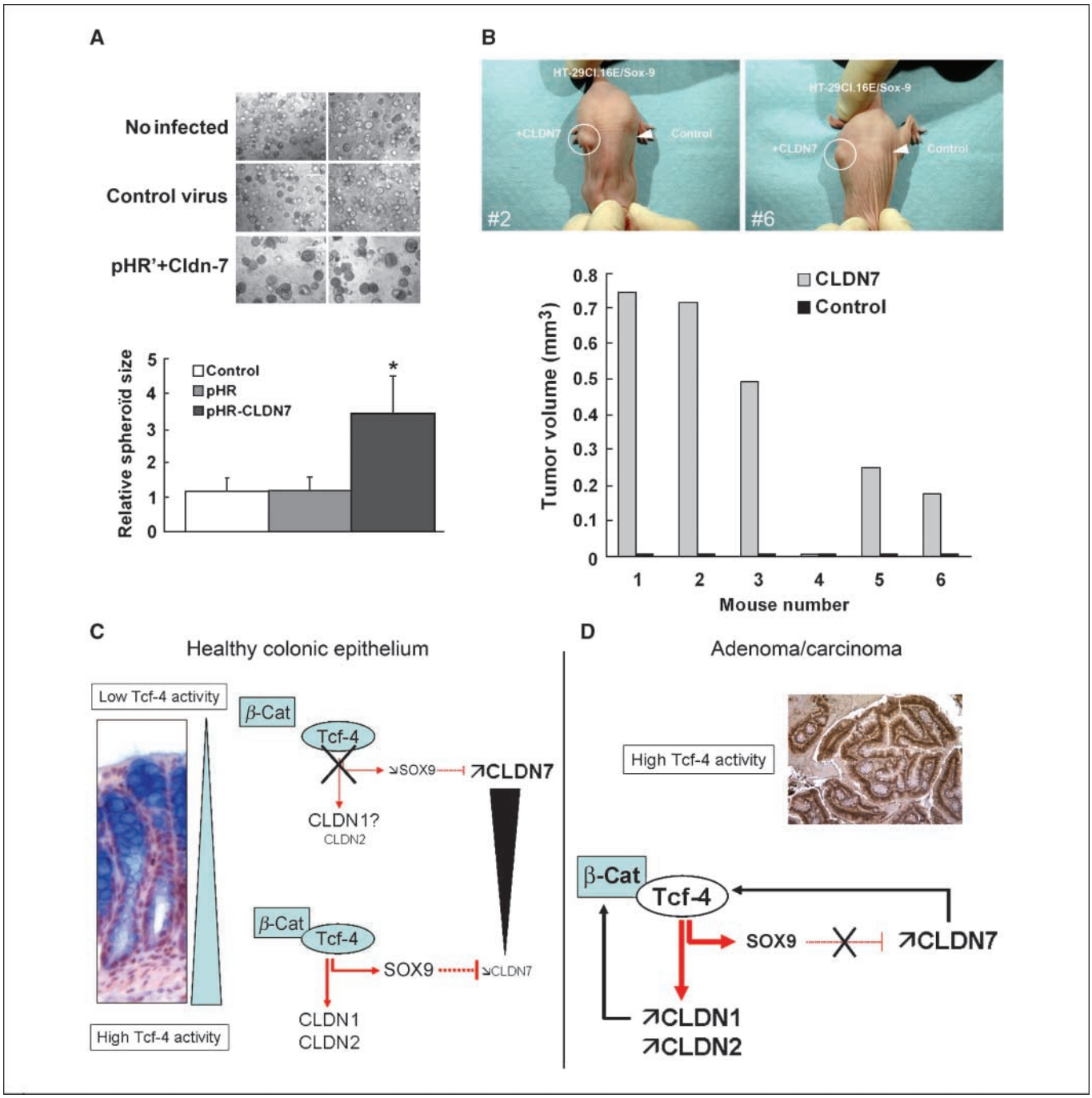

Figure 6. Claudin-7 overexpression enhances anchorage-independent growth and promotes tumor formation by CRC cell xenografts in immunodeficient mice. *A,* HT-29Cl.16E/Sox-9 cells were infected or not with control or claudin-7–expressing viral constructs, and grown in three-dimensional cultures as described (*bars*, 200 μm). Two representative photomicrographs per condition (*top*). The sizes of at least 100 spheroids were measured for each condition; *columns,* mean; *bars,* SE (*bottom*). *B,* six BALB/c nude mice were injected s.c. with 10^7 HT-29Cl.16E/Sox-9 cells infected or not with control (left flank) or claudin-7–expressing (right flank) viral constructs. They were provided with doxycyclin in the drinking water, and tumor size was measured 40 d after injection. Photographs show examples of tumors developed on the right flank only in mice no. 2 and no. 6, and the histogram summarizes tumor sizes in all six animals used. *C,* schematic summary of the Tcf-4/claudin-7 interaction in the healthy and tumoral colon. *Left,* the Tcf-4/Sox-9 axis down-regulates claudin-7 expression in the proliferative compartment of the healthy crypt, whereas Tcf-4 is inactive and Sox-9 very weakly expressed in differentiated cells, thus allowing claudin-7 expression. Claudin-1 and claudin-2 expression is positively regulated by Tcf-4 in the proliferative compartment, and claudin-1 expression is also detected in differentiated cells (12, 41). *Right,* in tumor cells, Tcf-4 activity is elevated but the claudin-7 repression mechanism is not or is poorly functional, maybe due to defective Sox-9 activity. This leads to the overexpression of claudin-7 in cancer cells, which in turn, enhances Tcf-4 activation. Elevated Tcf-4 activity also increases the expression of claudin-1 and claudin-2 (49), and the former has also been shown to stimulate Tcf-4 activity (10).

but rather concomitant with, the elevated expression of Tcf-4 targets such as Sox-9, c-Myc, and CD44 in human colorectal tumors. This result could indicate that claudin-7 expression becomes completely independent from Tcf-4 and Sox-9 in carcinomas, but our *in vitro* overexpression data rather seems to suggest that a downstream mediator of Tcf-4 activity as a claudin-7 regulator is dysfunctional within colorectal tumor cells. The identity of this mediator remains to be elucidated, although the low level of Sox activity in CRC cell lines and the capacity of experimental Sox-9 induction to restore some degree of claudin-7 repression in these cells suggest that this transcription factor could be poorly active in colorectal cancer cells.

Critically, our results also show that increased expression of claudin-7 in colorectal cancer cells leads to a loss of polarization and to an increase of β-catenin/Tcf-4 activity and proliferation in colorectal cancer cells, resulting in an enhancement of their tumor-forming ability *in vivo*. The relevance of claudins in the modulation of polarization and the development of invasive capabilities by tumor cells has been recently suggested in the case of claudin-1. The accumulation of this protein, induced by cathepsin L inhibition, was shown to correlate with a disorganization of intestinal cell layers (40). In addition, overexpression of this protein in colon tumor cells promoted the transition from an epithelial to mesenchymal phenotype and enhanced anchorage-independent growth (10), whereas claudin-1 silencing in oral squamous cell carcinoma strongly impaired their invasiveness (46). However, to our knowledge, this work presents the first demonstration of a role for claudin-7 in the regulation of polarization and tumor growth, suggesting that the strong overexpression of this protein detected in human colorectal cancer (13) and in gastric cancer (26) could be instrumental for tumor progression.

The stimulation of Tcf-4 transcriptional activity induced by claudin-7 in CRC cells also reflects the ability of claudin-7 overexpression to promote tumorigenesis. Although the capacity of the Tcf/Lef transcription factors to regulate claudin expression has been previously investigated (14, 37), the novel identification of a reciprocal modulation of Tcf-4 activity by a claudin family member corroborates the emerging string of data suggesting that TJ and TJ-related proteins are essential effectors of nuclear transcriptional programs (7). Selective disruption of TJ structure by cytoplasmic overexpression of the PDZ domains of ZO-1 (47) or by incubation with peptides mimicking an extracellular loop of occludin (48), was shown to induce an activation of the β-catenin/Tcf-4 complex via an up-regulation of β-catenin cytoplasmic availability. However, this mechanism is unlikely to play a part in the claudin-7–mediated regulation of Tcf-4 activity because we were unable to detect variations of nuclear β-catenin levels after claudin-7 modulation.

Because concomitant overexpression of claudin-7, CD44v6, EpCAM, and tetraspanin CO-029 was recently shown to correlate with enhanced metastasis formation (13), elucidating this mechanism and clarifying the molecular relationship between claudin-7 and its coexpressed partners in colorectal cancer will represent the next challenges to better understand how claudin-7 overexpression facilitates tumor progression and invasion.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

Received 10/9/2007; revised 3/10/2008; accepted 3/26/2008.

Grant support: INSERM (CreS no. 4CR04G), Association pour la Recherche contre le Cancer (ARC, no. 3563), and GEFLUC (F. Hollande); a Ph.D. Fellowship from CNRS-Lebanon (C. Darido), and the ARC (M. Buchert).

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The authors are very grateful to Dr. Y.I. Yeom for the generous gift of the ΔN87 β-catenin mutant constructs, to Dr. S. Pang for the claudin-7 lentiviral vector, to Prof. H. Clevers for providing us with the SOX/SAC constructs, and to Dr. J. Toguchida for the claudin-7 promoter in a PGV-B reporter vector. We are also grateful to U. Hibner and Delphine Haouzi for their help in setting up three-dimensional Matrigel cultures, N. Lautredou (Montpellier RIO Imaging Facility/CRIC) for her help with confocal microscopy, and A. Pélegrin for his help during nude mice xenograft studies.

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Cancer Res 2008;68:4258-4268.