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Autoregulation of E-cadherin expression by cadherin–cadherin interactions: the roles of β-catenin signaling, Slug, and MAPK

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Transcriptional repression of *E-cadherin*, characteristic of epithelial to mesenchymal transition, is often found also during tumor cell invasion. At metastases, migratory fibroblasts sometimes revert to an epithelial phenotype, by a process involving regulation of the E-cadherin–β-catenin complex. We investigated the molecular basis of this regulation, using human colon cancer cells with aberrantly activated β-catenin signaling. Sparse cultures mimicked invasive tumor cells, displaying low levels of E-cadherin due to transcriptional repression of *E-cadherin* by Slug. Slug was induced by β-catenin signaling and, independently, by ERK. Dense cultures resembled a differentiated epithelium with high levels of E-cadherin and β-catenin in adherens junctions. In such cells, β-catenin signaling, ErbB-1/2 levels, and ERK activation were reduced and Slug was undetectable. Disruption of E-cadherin–mediated contacts resulted in nuclear localization and signaling by β-catenin, induction of Slug and inhibition of *E-cadherin* transcription, without changes in ErbB-1/2 and ERK activation. This autoregulation of E-cadherin by cell–cell adhesion involving Slug, β-catenin and ERK could be important in tumorigenesis.

Introduction

Disruption of E-cadherin–mediated adhesion is considered a key step in progression toward the invasive phase of carcinoma (Behrens et al., 1992; Takeichi, 1993; Christofori and Semb, 1999). The mechanisms responsible for such changes in adhesion include mutations in the *E-cadherin* gene (*CDH1*) that compromise the adhesive capacity of E-cadherin (Hajra and Fearon, 2002), hypermethylation of the *E-cadherin* promoter (Graff et al., 1995; Hennig et al., 1995), or a combination of mutations in one allele with loss or inactivation (by DNA methylation) of the remaining allele (Berx et al., 1998; Machado et al., 2001). However, in many types of cancer, E-cadherin expression is lost without mutations in the gene (Hirohashi, 1998), owing to transcriptional repression of *E-cadherin* (Batlle et al., 2000; Cano et al., 2000; Comijn et al., 2001; Poser et al., 2001; Yokoyama et al., 2001; Hajra et al., 2002). Several transcription factors were implicated in such repression including a family of zinc finger proteins of the Slug/Snail family, δEF1/ZEB1, SIP-1, and the basic helix–loop–helix E12/E47 factor that interact with E-box sequences in the proximal *E-cadherin* promoter (Batlle et al., 2000; Cano et al., 2000; Grooteclaes and Frisch, 2000; Comijn et al., 2001; Perez-Moreno et al., 2001; Nieto, 2002; Bolos et al., 2003).

Transcriptional repression of *E-cadherin* and the associated morphological changes in cells also occur during epithelial to mesenchymal transition (EMT) in embryonic development, when epithelial cells move into new microenvironments and differentiate into various cell types, for example during neural crest cell migration from the neuroectoderm (Savagner, 2001; Thiery, 2002). Some of these processes, involving changes in E-cadherin, were also shown to involve activation of β-catenin signaling (Logan et al., 1999; Eger et al., 2000; Morali et al., 2001).

β-Catenin links the cadherin family of cell adhesion receptors to the actin cytoskeleton (Ben-Ze'ev and Geiger, 1998) and in addition, plays a key role in transduction of the Wnt

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Key words: cell adhesion; β-catenin; slug; ERK; tumorigenesis

Abbreviations used in this paper: EMT, epithelial to mesenchymal transition; RTK, receptor tyrosine kinases; WT, wild-type.

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signal, activating target gene expression in complex with Lef/Tcf transcription factors (Willert and Nusse, 1998). β-Catenin signaling operates at multiple stages during embryogenesis (Cadigan and Nusse, 1997) and maintains the proliferative compartment in adult intestinal epithelium (Batlle et al., 2002). Aberrant activation of β-catenin signaling is characteristic to early stages of colorectal carcinoma development (Bienz and Clevers, 2000; Polakis, 2000; Conacci-Sorrell et al., 2002a). This activation results from accumulation of β-catenin in the nuclei of epithelial cells owing to mutations in components of the degradation system (axin/conductin or APC) that regulates β-catenin turnover (Peifer and Polakis, 2000), or by stabilizing mutations in the NH_2 terminus of β-catenin (Korinek et al., 1997; Morin et al., 1997). Constitutive activation by β-catenin–Tcf/Lef complexes of target genes such as *cyclin D1* (Shtutman et al., 1999; Tetsu and McCormick, 1999) and *c-myc* (He et al., 1998), providing growth advantage to cells, are believed to contribute to the onset of oncogenesis.

Later stages in tumor development including acquisition of invasive and metastatic capacities by the tumor cells, require new cellular properties such as the ability to breakdown cadherin-mediated cell–cell contacts that keep normal epithelial cells adherent to each other. Activation of β-catenin signaling also contributes to these later changes by inducing other target genes, including metalloproteases (Brabletz et al., 1999; Crawford et al., 1999; Takahashi et al., 2002), ECM components (Gradl et al., 1999; Hlubek et al., 2001), and cell adhesion receptors such as *CD44* (Wielenga et al., 1999), *Nr-CAM* (Conacci-Sorrell et al., 2002b), and *uPAR* (Mann et al., 1999).

Recent studies of human colorectal cancer metastasis indicated that there are further similarities between EMT and colorectal cancer progression (Barker and Clevers, 2001). In particular, dynamic and reversible changes in E-cadherin and β-catenin localization were observed during colon cancer metastasis. These involve down-regulation of E-cadherin and nuclear localization of β-catenin at the invasive front, followed by reformation of a differentiated epithelial phenotype with junctional localization of E-cadherin and β-catenin at lymph node metastases (Brabletz et al., 2001).

In the present paper, we investigated the molecular basis of the reversible regulation of E-cadherin expression by cadherin–cadherin interactions and β-catenin signaling in colon carcinoma cells. We found that this regulation includes activation of *Slug* in sparse colon cancer cell cultures by two mechanisms: (1) involving transcriptional activation of *Slug* by the β-catenin–Tcf complex and (2) activation of the ERK pathway. When adherens junctions are established in dense cultures, ErbB-1, ErbB-2, and the ERK pathway become inactive, β-catenin is localized at adherens junctions, Slug expression is reduced, and *E-cadherin* transcription is induced. Antibody-mediated disruption of adherens junctions led to nuclear β-catenin localization and enhanced β-catenin signaling, induction of Slug and inhibition of E-cadherin expression. Our results point to an interplay between adherens junctions assembly and *E-cadherin* transcription mediated by junctional control of β-catenin signaling, and provide a molecular framework for the reversible repression of E-cadherin during colon cancer metastasis.

Results

Regulation of E-cadherin expression and β-catenin signaling by cell culture density

We have used human colon carcinoma cells with mutant APC (SW480), possessing stable wild-type (WT) β-catenin, and HCT116 and SW48 cells expressing β-catenin with stabilizing mutations at their NH_2 terminus. SW480 cells grown in sparse culture (6×10^3 cells/cm^2) for 2 d were characterized by weak and diffuse staining of E-cadherin but strong nuclear β-catenin localization (Fig. 1 A, sparse). In contrast, cells grown for 2 d as dense cultures (6×10^4 cells/

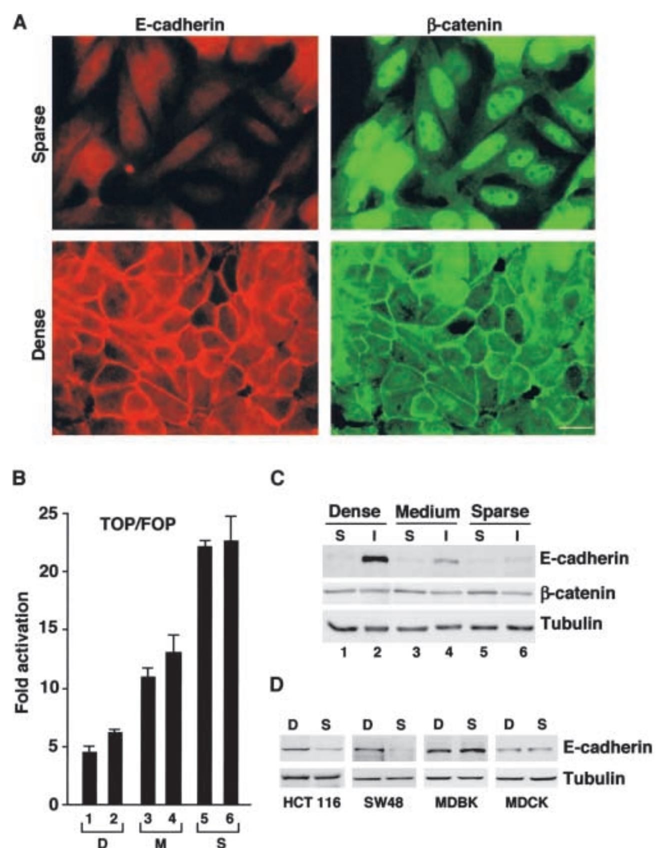

Figure 1. **Regulation of E-cadherin expression and β-catenin localization and signaling by cell density.** (A) SW480 cells were seeded from a semi-confluent culture dish at 6×10^3 cells/cm^2 (sparse) and 6×10^4 cells/cm^2 (dense), and after 2 d double stained for E-cadherin and β-catenin. (B) Cells cultured at different densities: lane 1, 6×10^4 cells/cm^2; lane 2, 3×10^4 cells/cm^2; lane 3, 2×10^4 cells/cm^2; lane 4, 1.5×10^4 cells/cm^2; lane 5, 10^4 cells/cm^2; and lane 6, 8×10^3 cells/cm^2 were transiently transfected with TOPFLASH (TOP) or FOPFLASH (FOP) reporters and fold activation was determined in duplicate dishes. S, sparse; M, medium; D, dense. The error bars represent the mean ± SD from triplicate plates. (C) Cells grown at different densities were fractionated into Triton X-100–soluble (S) and –insoluble (I) fractions and equal volumes (from equal numbers of cells) were analyzed by Western blotting for E-cadherin, β-catenin, and tubulin levels. (D) HCT116, SW48, colon cancer cells, and MDCK and MDBK normal epithelial cells were grown as sparse (6×10^3 cells/cm^2) and dense (6×10^4 cells/cm^2) cultures, and the levels of E-cadherin and tubulin were determined. Note induction of E-cadherin expression, relocalization of β-catenin to the membrane and inhibition of β-catenin–mediated transactivation in dense SW480 cells. Bar, 10 μm.

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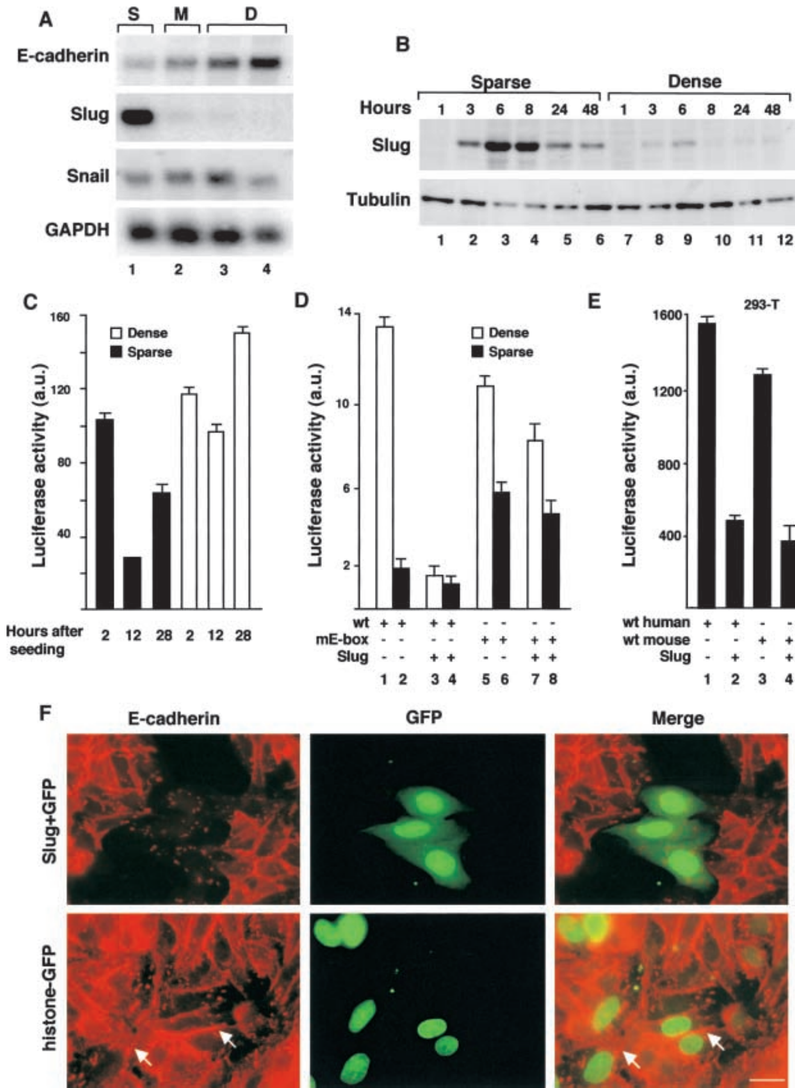

Figure 2. **Inhibition of E-cadherin expression in sparse cultures by Slug.** (A) SW480 cells were seeded at: lane 1, 6×10^3 cells/cm^2; lane 2, 2×10^4 cells/cm^2; lane 3, 4×10^4 cells/cm^2; and lane 4, 8×10^4 cells/cm^2), and the level of E-cadherin, Slug, Snail, and GAPDH poly(A)-RNA was determined by Northern blot hybridization with ^{32}P-labeled cDNA probes. (B) Cells from a confluent dish were seeded as sparse or dense cultures (as in Fig. 1 A), and at different times the level of Slug was determined by Western blot analysis of adherent cells using equal amounts of total protein. (C) Dense cultures were transfected with an *E-cadherin* promoter reporter and 14 h later the cells were seeded as sparse and dense cultures and promoter activity determined at different times after plating. (D) Sparse and dense cultures were transfected with WT mouse E-cadherin promoter reporter (wt), an E-box mutant promoter (mE-box), with or without a Slug cDNA plasmid and luciferase activity was determined. (E) Human and mouse WT E-cadherin promoter reporters were transfected into 293-T cells in the presence and absence of Slug and luciferase activity determined. (C–E) The error bars represent the mean ± SD from triplicate plates. (F) SW480 cells were transfected with a plasmid coding for both GFP and Slug, or histone-GFP, and stained for E-cadherin with rhodamine-labeled secondary antibody. Note that Slug expression correlated with reduced E-cadherin level, it inhibited the WT *E-cadherin* promoter and reduced E-cadherin expression in transfected cells. The arrows point to E-cadherin in adherens junctions of histone-GFP transfected cells. Bar, 10 μm.

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cm^2) displayed a more intense E-cadherin and β-catenin staining confined to cell–cell contacts, and a dramatic reduction in nuclear β-catenin (Fig. 1 A, dense). This relocalization of β-catenin from the nucleus to adherens junctions in dense cultures was associated with decreased β-catenin signaling shown by the reduction in activation of a transfected, β-catenin–LEF/TCF-responsive reporter plasmid (Fig. 1 B). Analysis of SW480 cultures seeded at different densities (by dilutions from a dense culture), indicated that in sparse cultures, where β-catenin was mainly localized in the nuclei, β-catenin signaling was maximal (Fig. 1 B, lanes 5 and 6); whereas with increasing culture density, β-catenin signaling was reduced (Fig. 1 B, lanes 1 and 2). Dense cultures also displayed a major increase in E-cadherin levels and its accumulation in the Triton X-100–insoluble membrane-cytoskeleton fraction (Fig. 1 C, compare lane 2 with lanes 4 and 6). Two other colon cancer cell lines, HCT116 and SW48, also showed an increase in E-cadherin level in dense compared with sparse cultures (Fig. 1 D), but the difference was less dramatic because unlike SW480 cells (Cano et al., 2000; Gottardi et al., 2001), these cells also accumulate E-cadherin in sparse cultures. In contrast, MDCK and MDBK normal epithelial cells expressing WT β-catenin, did not manifest such density-dependent regulation of E-cadherin (Fig. 1 D).

Down-regulation of E-cadherin in SW480 cells by Slug

To examine if the increase in E-cadherin results from elevated transcription, the levels of E-cadherin mRNA were determined by Northern blot hybridization of poly(A)-RNA from SW480 cells cultured for 2 d at different densities (Fig. 2 A, top). A significant elevation in E-cadherin mRNA occurred with increasing culture density (Fig. 2 A, top). Moreover, the transcriptional activity of a reporter plasmid containing the *E-cadherin* promoter was 5- to 7-fold higher in dense than in sparse cell cultures (Fig. 2 D, compare lane 1 with lane 2). Because transcription of *E-cadherin* is regulated, to a large extent, by the Snail/Slug family of repressors that bind to E-boxes in the proximal *E-cadherin* promoter (Hemavathy et al., 2000), we determined the mRNA levels of Snail and Slug in these SW480 cultures. Snail mRNA did not change significantly between sparse and dense cultures (Fig. 2 A, third from top), but the level of Slug mRNA was very high in sparse cultures (Fig. 2 A, second from top, lane 1) and undetectable in more dense cultures (Fig. 2 A, second from top, lanes 2–4), suggesting that Slug is involved in

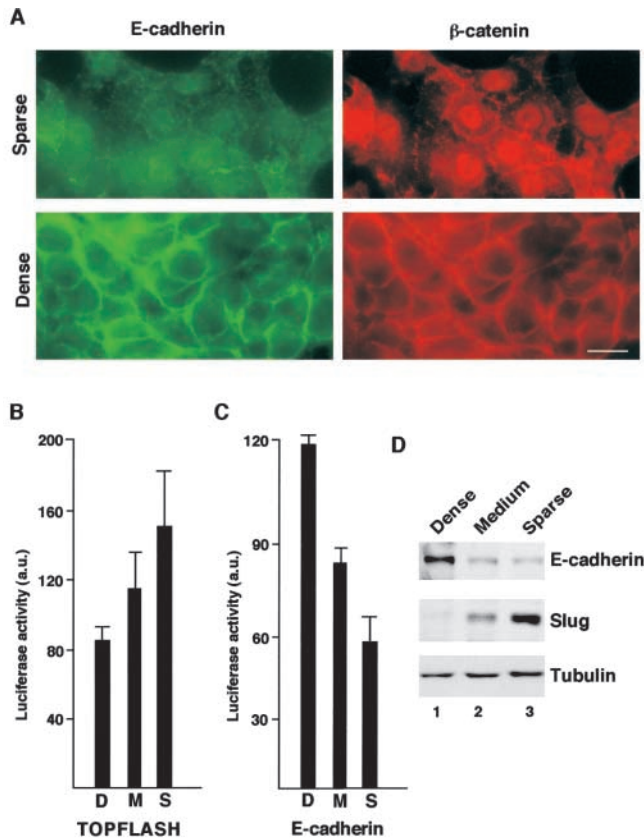

Figure 3. **Regulation of E-cadherin, Slug, and β-catenin localization and signaling by cell density in HCT116 cells.** (A) HCT116 cells were seeded as sparse and dense cultures (Fig. 1) and after 48 h the cells were double stained for E-cadherin and β-catenin. (B) Cells were seeded at different densities (as in Fig. 1A) and transfected with TOPFLASH, FOPFLASH, or (C) with the E-cadherin promoter reporter, and promoter activities were determined. (B and C) The error bars represent the mean ± SD from triplicate plates. (D) The levels of E-cadherin and Slug were determined by Western blot analysis 30 h after cell seeding at the densities indicated in Fig. 1 A. Bar, 10 μm.

transcriptional repression of *E-cadherin* in sparse SW480 cells. Expression of Slug was rapidly induced after seeding cells in sparse culture, peaking after 3–6 h (Fig. 2 B, lanes 1–6), whereas in dense cultures the low level of Slug induced between 3–6 h was lost at later times when cells established cell–cell contacts (Fig. 2 B, lanes 7–12). The kinetics of *E-cadherin* promoter response to culture density was determined by transfecting an *E-cadherin* reporter plasmid into dense cultures, followed by trypsinization of cells after 14 h and their seeding as sparse and dense cultures. E-Cadherin promoter activity decreased significantly at early times in sparse cultures, but not in dense cultures (Fig. 2 C, 12 h), in agreement with the early increase in Slug of sparse cultures (Fig. 2 B). At later times, when cells established contacts, *E-cadherin* promoter activity increased (Fig. 2 C, 28 h).

We also examined the organization and level of E-cadherin in HCT116 cells and the changes in β-catenin signaling, *E-cadherin* promoter activity and Slug expression, as a function of culture density (Fig. 3). We detected nuclear β-catenin and only weak E-cadherin staining in sparse cultures, whereas dense cultures presented stronger E-cadherin staining, but no distinct nuclear β-catenin (Fig. 3 A), resembling the results with SW480 cells (Fig. 1 A). β-Catenin signaling, measured by TOPFLASH activation (Fig. 3 B) and *E-cadherin* promoter activity (Fig. 3 C) were inversely regulated between sparse and dense cultures, as seen with SW480 cells (Fig. 1 B; Fig. 2 D). The level of Slug in HCT116 cells was high in sparse cells where E-cadherin levels were low, and very low in dense cultures where E-cadherin level was high (Fig. 3 D), as observed in SW480 cells.

To confirm that Slug can repress *E-cadherin* transcription in SW480 cells, a Slug cDNA was cotransfected with WT *E-cadherin* reporter, or with a mutant E-box *E-cadherin* promoter into sparse and dense SW480 cells. Slug was very efficient in repressing WT *E-cadherin* promoter activity in dense cultures (Fig. 2 D, compare lane 3 with lane 1), but could only weakly affect the mutant E-box promoter (Fig. 2 D, compare lane 7 with lane 5). The E-box mutant was more active than the WT promoter in sparse cultures (Fig. 2 D, compare lane 6 with lane 2), most probably because endogenous Slug could not bind and inhibit its activity. Moreover, transfected Slug was unable to suppress the mutant E-box promoter (Fig. 2 D, compare lane 8 with lane 6). Because the E-box mutant was still regulated (albeit weakly) by cell density (Fig. 2 D, compare lane 6 with lane 5), other mechanisms independent of the E-box may also be involved. Finally, Slug inhibited to a similar extent both the human and mouse *E-cadherin* promoter reporters in 293-T cells (Fig. 2 E).

To directly test whether Slug can affect endogenous E-cadherin levels in SW480 cells, a plasmid coding for both Slug and GFP was transfected and the cells were immunostained for E-cadherin. Slug expression resulted in dramatic reduction of E-cadherin levels (Fig. 2 F, top) and the morphology of the transfected cells changed to an extended fibroblastic shape. In contrast, the neighboring untransfected cells had an epithelial shape. Transfection of histone-GFP had no effect on cell morphology and the transfected cells maintained E-cadherin–containing junctions (Fig. 2 F, bottom, arrows).

Slug is activated by β-catenin/TCF signaling

Because Slug mRNA levels were high in sparse cultures of SW480 cells displaying nuclear β-catenin and strong β-catenin–mediated transactivation, whereas dense cultures lacked Slug and had low β-catenin signaling capacity (Figs. 1–3), we tested if the high Slug levels in sparse cultures result from activation of *Slug* by β-catenin signaling. Cotransfection of a mouse *Slug* promoter reporter together with stabilized S33Y β-catenin into 293-T cells showed activation of the *Slug* promoter by cotransfected β-catenin (Fig. 4 A, compare lane 2 with lane 1), and also by endogenous β-catenin in SW480 cells (Fig. 4 B, lane 1). In contrast, *Snail* promoter activity was not induced by β-catenin (Fig. 4 A, lanes 5 and 6). In 293 cells, transient transfection of GFP-tagged Slug very effectively reduced the endogenous E-cadherin (Fig. 4 C, compare lane 3 with lane 1), whereas transfection of GFP-Snail had only a mild effect (Fig. 4 C, compare lane 2 with lane 1). Dominant negative Tcf blocked activation of the *Slug* promoter (Fig. 4 A, compare lane 3 with lane 2; Fig. 4 B, compare lane 2 with lane 1), similar to the cytoplasmic domain of cadherin that seques-

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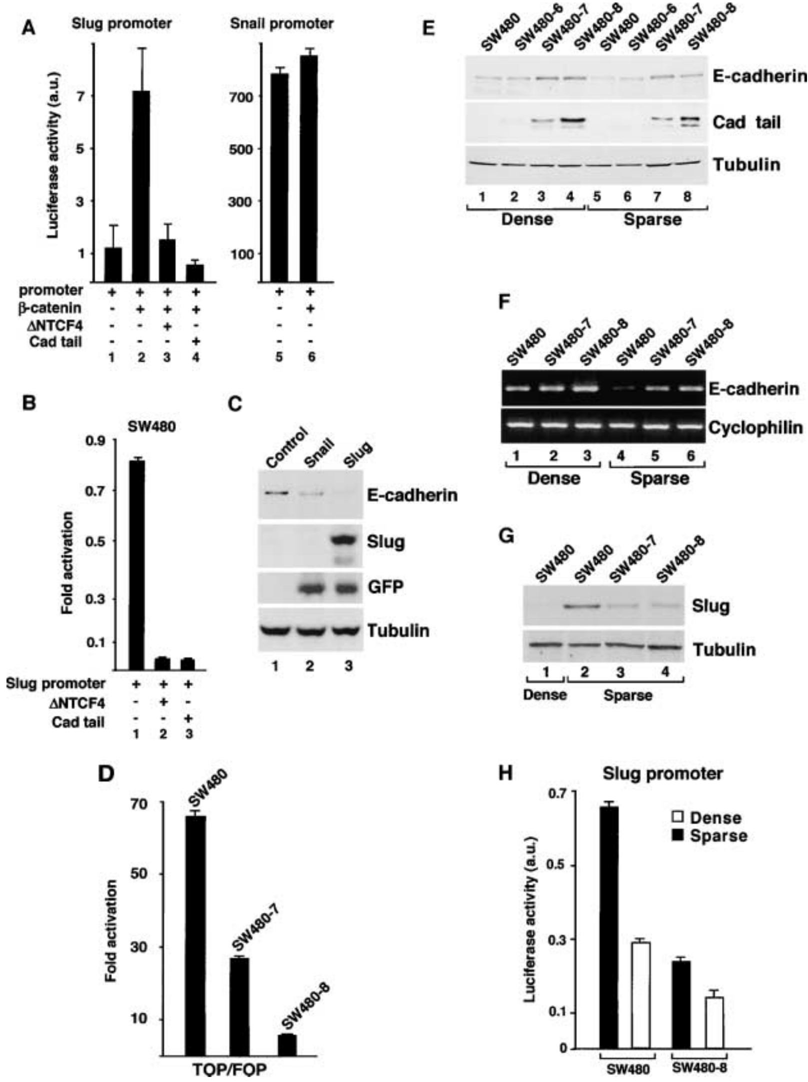

Figure 4. **Activation of *Slug* transcription by β-catenin–TCF signaling.** (A and B) Activation of mouse *Slug*, but not *Snail*, promoter by β-catenin in 293-T (A), and of the *Slug* promoter in SW480 cells (B). Inhibition of *Slug* promoter activation by dominant negative TCF (ΔNTCF4) and the cytoplasmic tail of cadherin (Cad tail). (C) Transfection of Slug, and to a lesser extent Snail, into 293-T cells reduces endogenous E-cadherin levels. Cells were transfected with a plasmid coding for Slug and GFP, or Snail and GFP, and the levels of GFP, Slug, and E-cadherin were determined by Western blotting. (D) Decreased β-catenin/TCF–mediated transactivation in SW480 clones (SW480–7 and SW480–8) stably expressing the cadherin tail. (E) SW480 clones expressing the cadherin tail displayed elevated E-cadherin protein, (F) increased E-cadherin RNA (by RT-PCR), and (G) decreased Slug protein and (H) lower Slug promoter activity. (A, B–D, and H) The error bars represent the mean ± SD from triplicate plates.

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ters β-catenin from binding to Tcf (Fig. 4 A, compare lane 4 with lane 2; Fig. 4 B, compare lane 3 with lane 1).

Next, we asked if β-catenin signaling is essential for inhibition of E-cadherin expression by Slug. We used SW480 clones stably expressing varying levels of the cadherin cytoplasmic domain (Shtutman et al., 1999) and displaying decreased β-catenin–dependent transactivation (Fig. 4 D). Clones expressing high levels of the cadherin tail (SW480–7 and SW480–8), had more E-cadherin than control cells, or clone SW480–6, that only accumulated very low levels of cadherin tail (Fig. 4 E, compare lanes 7 and 8 with lanes 5 and 6). In agreement with the changes in E-cadherin protein level, E-cadherin RNA levels (Fig. 4 F) were also higher in clones SW480–7 and SW480–8 than in control SW480 cells. This was more evident in sparse cultures (Fig. 4 E, compare lanes 5 and 6 with lane 4). Inhibition of β-catenin signaling resulted in decreased Slug levels in SW480–7 and SW480–8 compared with parental SW480 cells (Fig. 4 G, compare lanes 3 and 4 with lane 2). Transcriptional activity of the *Slug* promoter in SW480–8 cells was also lower than in control cells (Fig. 4 H).

These results suggest that the strong β-catenin–Lef/Tcf signaling in sparse cultures induced the *Slug* gene resulting in repression of *E-cadherin* transcription. Inhibition of β-catenin signaling, by the cadherin cytoplasmic tail, reduced Slug expression de-repressing the *E-cadherin* gene, and leading to increased E-cadherin levels.

ERK activation regulates E-cadherin and Slug expression

Because E-cadherin regulation and induction of *Slug* were shown to involve the MAPK (ERK) pathway (Boyer et al., 1997; Weng et al., 2002), we investigated whether the cell culture density–related regulation of E-cadherin and Slug in SW480 cells involves ERK activation. We detected very high levels of activated ERK in sparse cultures, compared with dense cultures (Fig. 5 A, third from top). Inhibition of the ERK pathway by PD98059 (Fig. 5 A, third from top, compare lane 2 with lane 1) induced an increase in E-cadherin levels of sparse cultures (Fig. 5 A, top, compare lane 2 with lane 1), but had no effect in dense cultures that had no detectable activated ERK (Fig. 5 A, top, second from top, and third from top, lanes 3 and 4). E-Cadherin RNA level was also induced in the presence of PD98059, especially in sparse cultures (Fig. 5 B, lanes 3 and 4). We also tested the ability of ERK to affect *E-cadherin* promoter

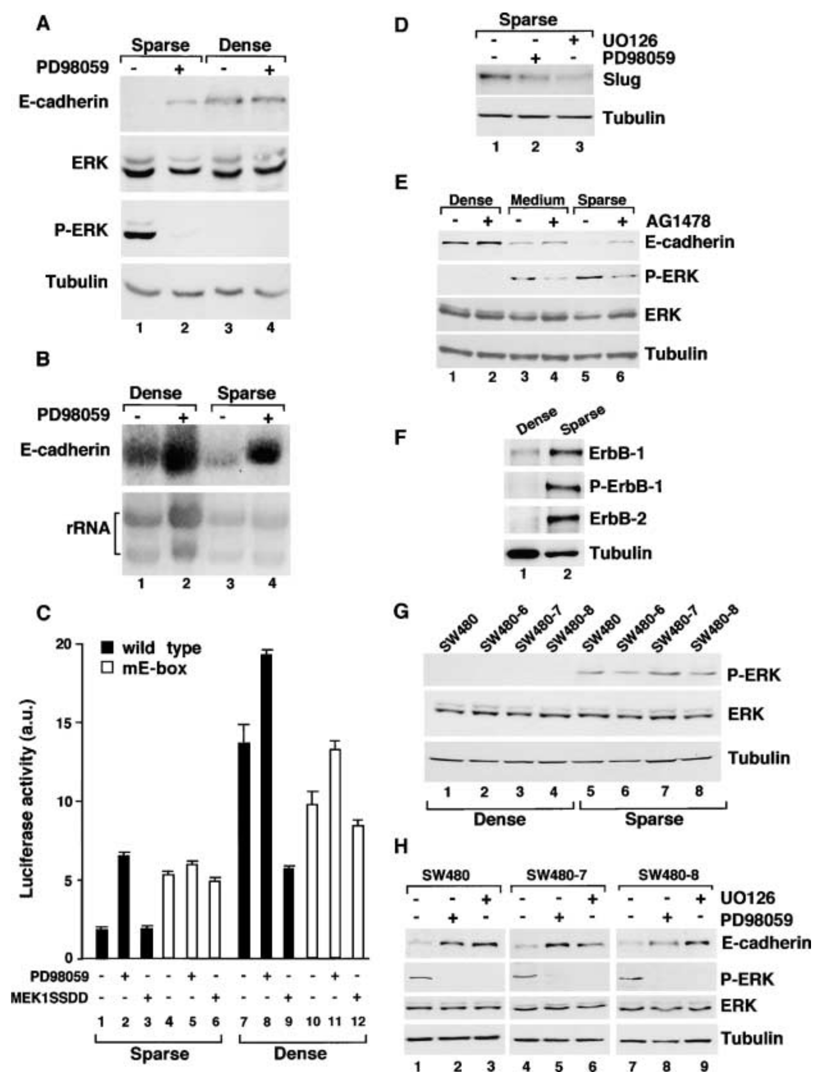

Figure 5. **ERK activation and β-catenin signaling can independently regulate E-cadherin expression via Slug.** (A) Activation of ERK was inhibited in sparse and dense cultures by incubating cells for 24 h with PD98059 and the level of E-cadherin, total ERK, P-ERK, and tubulin were determined by Western blot analysis. (B) The effect of PD98059 on E-cadherin RNA levels was determined by Northern blot hybridization in sparse and dense cultures of SW480 cells. (C) The effect of PD98059 and activated ERK (MEK1SSDD) on WT E-cadherin and E-box mutant promoter (mE-box) activity was determined in sparse and dense cultures. The error bars represent the mean ± SD from triplicate plates. (D) Inhibition of ERK activation by PD98059 and UO126 in sparse cultures reduced the level of Slug. (E) The RTK inhibitor tyrphostin AG1478 inhibited ERK activation and elevated E-cadherin expression in sparse cultures but not in dense cultures. (F) The levels of ErbB-1 and ErbB-2 and the phosphorylation of ErbB-1 (P-ErbB-1) were determined in sparse and dense cultures. (G) SW480 clones expressing the cytoplasmic tail of cadherin displayed unaltered P-ERK levels compared with control SW480 cells and, (H) inhibition of ERK activation in these cells by PD98059 and UO126 elevated E-cadherin levels.

activity and found that PD98059 enhanced it in sparse cultures (Fig. 5 C, compare lane 2 with lane 1), but had a weaker effect in dense cultures (Fig. 5 C, compare lane 8 with lane 7). Transfection of constitutively active ERK (MEK1SSDD) inhibited *E-cadherin* promoter activity in dense cultures (Fig. 5 C, compare lane 9 with lane 7), but had no effect in sparse cultures (Fig. 5 C, compare lane 3 with lane 1) because these cells already displayed high levels of activated endogenous ERK (Fig. 5 A, third from top, lane 1). Interestingly, MEK1SSDD did not affect the E-box mutant *E-cadherin* promoter (Fig. 5 C, compare lane 6 with lane 4), which was very active also in sparse cultures (Fig. 2 D, compare lane 6 with lane 2), indicating that activated ERK regulates the *E-cadherin* promoter also via the E-box domain. To ask if ERK affects *E-cadherin* transcription by inducing Slug, sparse cultures were treated with two different inhibitors of the ERK pathway (PD98059 and UO126), and both were found to reduce Slug levels (Fig. 5 D, compare lanes 2 and 3 with lane 1). These results suggest that activated ERK can repress E-cadherin expression in sparse cells, most probably by inducing Slug that inhibits *E-cadherin* transcription.

Because ERK induction usually results from activation of receptor tyrosine kinases (RTK), we examined whether their inhibition by tyrphostin AG1478 elevates E-cadherin protein in sparse cultures displaying activated ERK. AG1478 was most effective in increasing E-cadherin in sparse cells (Fig. 5 E, compare lane 6 with lane 5) and semi-confluent cultures (Fig. 5 E, compare lane 4 with lane 3), where it reduced ERK activation, but not in dense cultures lacking activated ERK (Fig. 5 E, lanes 1 and 2). Consistent with these observations, the levels and activity of the EGFR family members ErbB-1 and ErbB-2/Neu were high in sparse, but very low in dense cultures (Fig. 5 F). We also examined if soluble factors secreted by sparse cultures are involved in stimulating RTK, or whether growth inhibitors secreted by dense cultures inhibited their activity. Coverslips of sparse and dense cells were placed in the same dish and, in other experiments, conditioned medium from sparse and dense cultures were exchanged. Such experiments did not reveal changes in E-cadherin expression either in sparse or dense cultures (unpublished data).

β-Catenin signaling and ERK activation independently inhibit E-cadherin expression

Next, we asked if the mechanisms involving ERK activation and β-catenin signaling in the regulation of E-cadherin are

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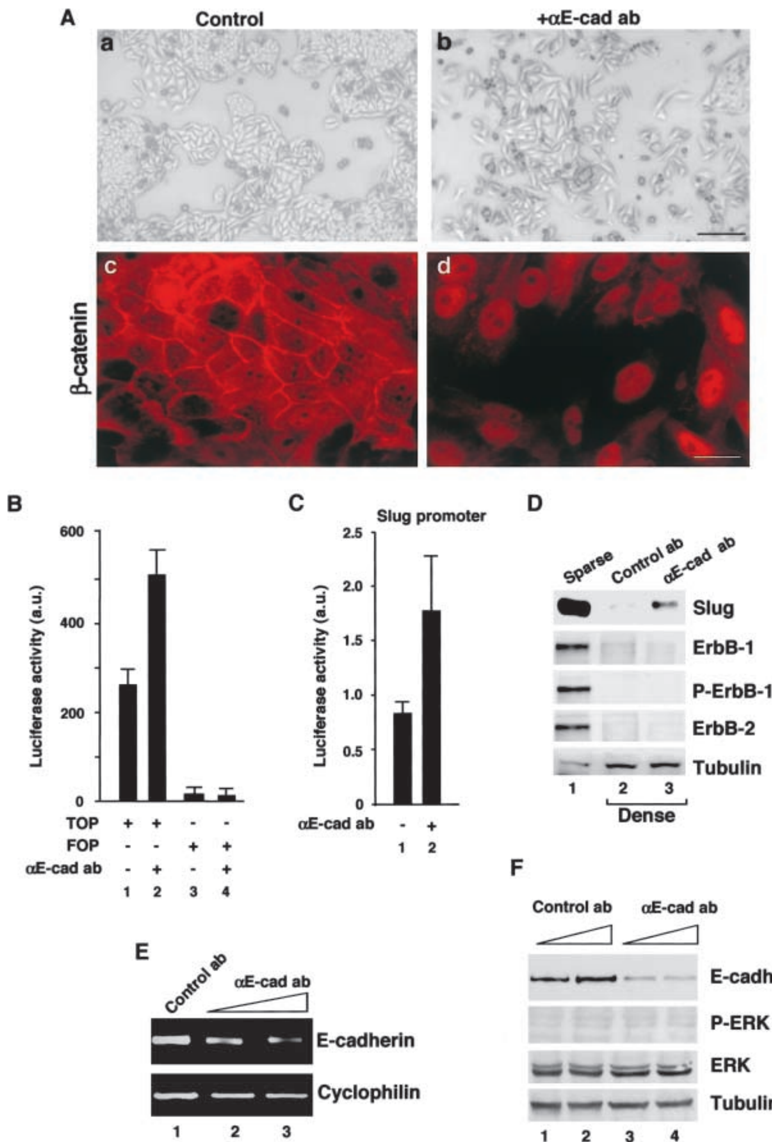

Figure 6. **Inhibition of E-cadherin–E-cadherin interactions in dense SW480 cultures induces nuclear localization and signaling by β-catenin, elevates Slug expression and reduces E-cadherin levels.** (A) SW480 cells were seeded as dense cultures in the presence of a polyclonal anti–E-cadherin antibody (αE-cad), or control antibody, and cell morphology and the organization of β-catenin (by anti–β-catenin antibody staining) were determined. (B) Cells were first transfected with TOPFLASH, FOPFLASH, or the Slug reporter plasmid and seeded in the presence of 1:50 dilution of anti–E-cadherin or control antibody. (B) The activities of TOPFLASH (TOP) or control, FOPFLASH (FOP), and (C) the Slug promoter were determined. (B and C) The error bars represent the mean ± SD from triplicate plates. (D) The levels of Slug, ErbB-1, P-ErbB-1, and ErbB-2, were determined in sparse and dense cultures and in dense cultures incubated with anti–E-cadherin antibody. (E) E-cadherin RNA levels were determined by RT-PCR. (F) The levels of E-cadherin protein, total ERK, activated ERK (P-ERK) and tubulin were determined by Western blot analysis of lysates from cells incubated with 1:10 (lanes 2 and 4) and 1:50 dilutions (lanes 1 and 3) of the control and anti–E-cadherin antibodies. Note that antibody-mediated inhibition of E-cadherin–mediated adhesion resulted in nuclear localization of β-catenin, elevation in Slug, and reduction in E-cadherin expression, but no changes in ErbB-1/ErbB-2 and ERK activation. Bars: (A, d) 10 μm; (A, b) 60 μm.

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linked. We determined the level of activated ERK in SW480 clones expressing the cadherin tail and found no significant differences in P-ERK between parental cells and clones expressing the cadherin tail, either in sparse or dense cultures (Fig. 5 G). Moreover, in clones expressing the cadherin tail (and therefore having reduced β-catenin signaling), inhibition of ERK by PD98059 or UO126 increased E-cadherin levels as observed in control cells (Fig. 5 H, lanes 2 and 3; 5 and 6; and 8 and 9, compare with lanes 1, 4, and 7, respectively). This suggests that when β-catenin signaling is inhibited, the blocking of ERK still results in E-cadherin elevation, indicating that ERK activation and β-catenin signaling can independently repress E-cadherin.

Cadherin–cadherin interactions increase E-cadherin levels

We also determined if the assembly of adherens junctions in dense SW480 cultures is involved in inducing E-cadherin expression. To inhibit E-cadherin–dependent adherens junctions assembly in long term cultures (48 h), dense cultures were seeded in the presence of a polyclonal antibody against the extracellular domain of E-cadherin to block cadherin–cadherin interactions. Such cells had altered colony morphology with scattered cells, compared with cells cultured with control antibody that were organized in colonies (Fig. 6 A, compare panel b with panel a). The organization of β-catenin also underwent a dramatic change, opposite to that described in Fig. 1 A: instead of localizing to cell–cell contacts (Fig. 6 A, c), β-catenin relocalized to the nuclei of cells with only little β-catenin found in adherens junctions (Fig. 6 A, d). Cells that were first transfected with TOPFLASH, or the *Slug* promoter reporter, and then seeded in the presence of anti–E-cadherin antibody, displayed increased β-catenin signaling and *Slug* promoter activity compared with control (Fig. 6, B and C, compare lane 2 with lane 1, respectively). Slug protein level was higher in dense cultures incubated with the antibody (Fig. 6 D, compare lane 3 with lane 2), but was significantly lower than in sparse cultures (Fig. 6 D, compare lane 3 with lane 1). E-Cadherin RNA and protein levels were also reduced in cells incubated with anti–E-cadherin antibody (Fig. 6 E, compare lanes 2 and 3 with lane 1; Fig. 6 F, compare lanes 3 and 4 with lanes 1 and 2). Inhibition of E-cadherin expression did not in-

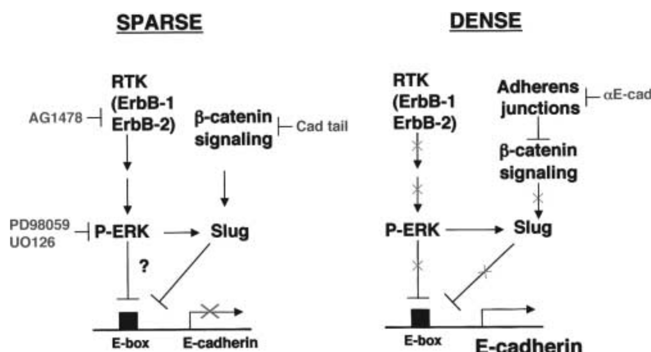

Figure 7. **Mechanisms of E-cadherin regulation in sparse and dense cultures of colon cancer cells.** In sparse cultures, E-cadherin expression is suppressed by two different mechanisms. One involves activation of ERK (P-ERK) by receptor tyrosine kinases (RTK), such as ErbB-1 and ErbB-2. ERK activation induces *Slug* that inhibits E-cadherin expression by binding to the E-box of *E-cadherin*. The other pathway includes *Slug* induction by the β-catenin–TCF pathway. In dense cultures, the ERK pathway is inactive, the levels of ErbB-1, ErbB-2, and Slug are repressed and E-cadherin expression increases. This leads to recruitment of nuclear β-catenin to adherens junctions together with E-cadherin and the inhibition of β-catenin signaling. This further reduces Slug expression and relieves the inhibition on *E-cadherin* transcription resulting in elevated E-cadherin expression.

volve an induction in ErbB-1 and ErbB-2 levels or activity (Fig. 6 D, ErbB-1, P-ErbB-1, and ErbB-2, compare lanes 2 and 3 compare with lane 1, respectively) or ERK activity (Fig. 6 F, second from top) that remained very low. These results suggest that β-catenin signaling and ErbB-1/ErbB-2-ERK activation can independently regulate Slug and E-cadherin expression, and are probably both required for full regulation by a positive feedback mechanism driven by RTK and the assembly of adherens junctions (Fig. 7).

Discussion

EMT and tumor cell metastasis are believed to share common properties including dismantling of cadherin-mediated cell–cell junctions (characteristic of epithelia), acquisition of a fibroblastic phenotype, the ability to invade into the extracellular environment, and movement to distant sites (Savagner, 2001; Thiery, 2002). During development, classical examples of EMT, including gastrulation and neural crest cell migration, give rise to motile cell populations that differentiate later into various epithelial structures and other cohesive cell structures, including muscular and neural cells that express specialized cell–cell adhesions (for review see Savagner, 2001). Reversion to an epithelial morphology of invasive cancer cells was recently demonstrated during human colorectal cancer development. Such cancer cells were shown to first switch from a tubular and epithelial organization into a fibroblastic phenotype at the invasive front of the primary tumor, followed by "re-differentiation" into tubular epithelial structures at lymph node metastases (Brabletz et al., 2001).

In this paper, we determined the molecular basis of the changes in E-cadherin expression and the associated alterations in β-catenin localization and signaling in colon carcinoma cells displaying activating mutations in β-catenin signaling (owing to mutations in APC or in β-catenin), when the cells regained an epithelial phenotype from a more fibroblastic one. Sparse cultures of SW480 and HCT116 cells resembled cells at the invasive front of colon carcinoma, characterized by extensive nuclear β-catenin, high levels of β-catenin–Tcf signaling and very low levels of E-cadherin (Brabletz et al., 2001). This resulted from transcriptional repression of the *E-cadherin* gene by two different pathways (Fig. 7). One, involving activated RTK of the EGFR family (ErbB-1 and ErbB-2) leading to activation of ERK that resulted in the induction of Slug, a repressor of *E-cadherin*. The other pathway involved induction of *Slug* transcription by the β-catenin–Tcf complex, indicating that *Slug* might be a target gene of β-catenin signaling. This view is supported by the presence of two putative Lef/Tcf sites in the mouse *Slug* promoter (unpublished data), inhibition of *Slug* promoter activation by dominant negative Tcf (Fig. 4, A and B), and the reported Lef/Tcf binding sequence in the *Xenopus Slug* promoter that is involved in neural crest cell determination (Vallin et al., 2001).

Transcriptional repression of E-cadherin induced by activated ERK or β-catenin signaling involved, in both cases, induction of Slug. Moreover, transfection of Slug into SW480 cells abolished E-cadherin expression (Fig. 2 F) and there was a correlation in the kinetics of Slug induction in sparse cultures (Fig. 2 B) and transcriptional repression of the *E-cadherin* promoter (Fig. 2 C). When ErbB-1 and ErbB-2 levels and activities were reduced (and ERK signaling inhibited), the activity of WT *E-cadherin* promoter was elevated, whereas that of an E-box mutant was not (Fig. 5 C). This implies that the repressive effects of ERK on the *E-cadherin* promoter operated via E-box elements in this promoter where members of the Snail/Slug family bind. Because Slug expression was high in sparse cultures and absent in dense cultures, but was rapidly induced upon dispersion of dense cultures after trypsinization into sparse cultures (Fig. 2 B), Slug was most probably responsible for down-regulating *E-cadherin* transcription in sparse SW480 cell cultures. This view is supported by our finding that the *Slug*, but not the *Snail*, promoter was activated by β-catenin (Fig. 4 A) and Slug transfection was effective, whereas that of Snail was weak, in reducing endogenous E-cadherin in 293 cells (Fig. 4 C).

Interestingly, inhibition of the integrin-linked kinase pathway in colon cancer cells, which led to suppression of β-catenin signaling, also induced E-cadherin expression and repressed *Snail* promoter activity (Tan et al., 2001). Because β-catenin signaling is not involved in *Snail* promoter regulation (Fig. 4 A), the mechanisms involved in Snail regulation by integrin-linked kinase in colon cancer cells are yet unknown.

Previous studies suggested a link between increased β-catenin signaling and down-regulation of E-cadherin in MDCK (Reichert et al., 2000) and RK3E rat kidney epithelial cells (Weng et al., 2002). In RK3E cells expressing a transfected, inducible chimeric β-catenin construct, the elevation in β-catenin and ERK activation resulted in down-regulation of E-cadherin (Weng et al., 2002). These studies support our observation that β-catenin–Tcf signaling and ERK activation reduce E-cadherin levels in sparse SW480 colon cancer cells. We have shown, in addition, that this

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regulation operates by the induction of Slug. Although induction of β-catenin leads to ERK activation in RK3E cells, in SW480 colon cancer cells the activation of ERK and β-catenin signaling could operate independently of each other to trigger Slug expression (Figs. 5–7). This difference may have resulted from the different cells used (normal epithelial versus carcinoma cells), or from using a transfected β-catenin chimera in RK3E cells, in contrast to signaling by WT endogenous β-catenin in SW480 cells.

Dense cultures of SW480, HCT116, and SW48 colon cancer cells resembled the differentiated areas of tubular organization in colon carcinoma, at both the primary tumor site and lymph node metastases (Brabletz et al., 2001), displaying increased junctional organization of E-cadherin and β-catenin. We found that such dense cultures did not present activated ERK nor expressed Slug, thereby relieving the repression on E-cadherin transcription and allowing E-cadherin accumulation. An association between cell culture density and MAPK (ERK) activity could reflect both in vitro and in vivo a modulation in RTK activity, or expression of the ErbB-1/ErB-2 family (Fig. 5 F), as also described for other cultured carcinoma cells (Takahashi and Suzuki, 1996; Savagner et al., 1997).

The increase in E-cadherin levels in dense cultures resulted in relocalization of β-catenin from the nucleus to a membranal complex with E-cadherin in adherens junctions and reduction in β-catenin–Tcf/Lef signaling. Dense cultures of SW480 cells had a lower percentage of cells in S-phase compared with sparse cultures (unpublished data), in agreement with recent studies suggesting that E-cadherin (via its cytoplasmic domain) suppresses cell growth by inhibiting β-catenin signaling (Gottardi et al., 2001; Stockinger et al., 2001). An earlier paper also demonstrated that disruption of E-cadherin–mediated cell–cell adhesion, by an antibody to E-cadherin, induces proliferation in colon and other cancer cells (St Croix et al., 1998). These findings are in contrast to the increase in proliferation markers observed in E-cadherin-positive differentiated tubular colon carcinoma cells and the diminished level of such markers in invasive colon cancer cells displaying nuclear β-catenin (Brabletz et al., 2001). These differences most probably result from the different microenvironment around tumor cells in vivo as compared with cells cultured in vitro.

In dense cultures, the increased in E-cadherin could be inhibited when the cells were grown in the presence of anti–E-cadherin antibody (Fig. 6, E and F) that blocked cadherin–cadherin interactions. Also, Slug was rapidly induced in sparse cultures after dense cell culture dispersion by trypsinization (Fig. 2 B). The relocalization of β-catenin to nuclei and induction of β-catenin signaling activity and Slug expression (albeit partial; Fig. 6 D, compare lanes 2 and 3 with lane 1) occurred without a change in ErbB-1/ErB-2 and ERK activation. This demonstrated the importance of β-catenin signaling and Slug in regulating E-cadherin expression and their ability to function (at least in part) independently of the ERK pathway.

β-Catenin–Tcf signaling is required in the proliferative compartment of intestinal epithelium at the bottom of crypts where cells maintain their epithelial phenotype (van de Wetering et al., 2002). Aberrant activation of β-catenin signaling results in disruption of the balance between the proliferative and differentiated compartments leading to intestinal polyp formation and later, to invasion into the stroma. It remains to be determined whether such hyper activation of β-catenin target genes includes the activation of *Slug*. We found that Slug induction was only apparent in very sparse colon cancer cell cultures (Fig. 2, A and B; Fig. 3, A and D) displaying the highest level of β-catenin signaling (Fig. 1 B; Fig. 3 B) and lacking adherens junctions (Fig. 1 A), similar to cells at the invasive front of colon tumors (Brabletz et al., 2001). Such strong β-catenin signaling and additional signals (like activation of the EGFR–ERK pathway) might both be necessary to induce *Slug* during colon cancer development.

Our description of E-cadherin regulation by β-catenin–Tcf signaling by controlling *Slug* transcription and involving cadherin mediated cell–cell interactions, unraveled an important aspect of the molecular pathways that could govern human colon cancer development. A recent paper showed that such inverse relationship between β-catenin nuclear localization and signaling and down-regulation of E-cadherin expression, is also an integral part of hair follicular bud development (Jamora et al., 2003) that involves and interplay between Wnt and BMP signals. Therefore, this link between cell adhesion, signal transduction, and the regulation of transcription by the cadherin–β-catenin system appears to have implications for both epithelial development and cancer. Future studies using this model system will allow addressing the relationship(s) of the cadherin–catenin system with RTK and downstream components of the MAPK pathway, and the conditions responsible for triggering *Slug* repression when cells establish contacts and acquire an epithelial phenotype.

Materials and methods

Cell lines, cell culture, and transfections

SW480, 293-T, HCT116, MDBK, and MDCK cells were grown in DME with 10% bovine calf serum. SW480 cells expressing the NH_2-cadherin cytoplasmic tail (Shtutman et al., 1999) were cultured with 100 μg/ml hygromycin B, and SW48 cells in McCoy's 5A medium with 10% bovine calf serum. A semi-confluent culture was seeded into 35-mm dishes as dense (6×10^4; 3×10^4 cells/cm^2), medium (2×10^4; 1.5×10^4 cells/cm^2), and sparse (8×10^3; 6×10^3 cells/cm^2) cultures from one original dish. After 48 h, total cell lysates were prepared for Western or Northern blot analysis. In some cases a Triton X-100–soluble and –insoluble fraction was first prepared (Sadot et al., 1998). Cells were also incubated with the ERK inhibitors PD98059 (25 μM) and UO126 (15 μM), and the RTK inhibitor AG1478 (500 nM; Calbiochem), a gift from R. Seger, Weizmann Institute of Science, for the last 24 h before cell harvesting. To disrupt adherens junctions, SW480 cells were incubated for 48 h with 1:10 or 1:50 dilutions of polyclonal antibody against the extracellular domain of human E-cadherin, provided by M. Wheelock (University of Nebraska, Omaha, NE). Transient transfections into SW480 and 293-T cells were performed with lipofectamin (GIBCO BRL). For transactivation assays, 0.5 μg of β-galactosidase plasmid was cotransfected with 1 μg of reporter plasmids and 3.5 μg β-catenin S33Y, or the Slug construct, in duplicate plates; and after 48 h, luciferase and β-galactosidase activities were determined as described previously (Shtutman et al., 1999).

Plasmids

The WT and E-box mutant mouse *E-cadherin* promoters provided by A. Cano (Instituto de Investigaciones Biomedicas CSI-UAM, Madrid, Spain) were subcloned into the BglII-SacI sites of pGL3 fused to the luciferase reporter gene. A 2.8-kb genomic fragment containing the mouse Slug pro-

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moter was cloned from a mouse embryonic library prepared by Y. Yamada (National Institutes of Health, Bethesda, MD) using a mouse cDNA probe (Savagner et al., 1997). The promoter region was sequenced (unpublished data) and cloned into the BglII-KpnI sites of pGL3 fused with luciferase. The human *E-cadherin* and *Snail* promoters cloned into pGL3 were from A.G. de Hereros (Universitat Pompeu Fabra, Barcelona, Spain). TOPFLASH, FOPFLASH, and dominant negative TCF4 (ΔNTCF4) were provided by H. Clevers and M. van de Wetering (Utrecht University Medical Center, Utrecht, Netherlands). Human Slug, a gift from T. Ip (University of Massachusetts Medical School, Worcester, MA) and Snail cDNAs were cloned into the pTracer expression vector encoding for GFP under the control of an independent promoter (unpublished data). The mutant β-catenin S33Y (Shtutman et al., 1999) and the plasmid coding for the cytoplasmic domain of E-cadherin (E-cad tail) were described previously (Sadot et al., 1998). The MEK1SSDD plasmid was provided by J. Pouyssegur (Institute of Signaling, Developmental Biology and Cancer Research, Nice, France) and B. Boyer (Institute Curie, Paris-Sud, France).

RNA analysis

Northern blot hybridization was performed using 30 μg total RNA, or polyadenylated RNA isolated from 300 μg total RNA using the PolyA Tract system IV (Promega). Membranes were hybridized with ^{32}P-labeled human E-cadherin cDNA, a gift from J. Behrens (Max-Delbruck-Center for Molecular Medicine, Berlin, Germany), ^{32}P-labeled mouse Snail cDNA, a gift from A. Cano, with the 5′-UTR of the human Slug gene pCRII.H.Slug.P64–41, and a cDNA to GAPDH. RT-PCRs for E-cadherin and cyclophilin A were performed using the primers and PCR conditions described previously (Batlle et al., 2000).

Immunofluorescence

Cells cultured on glass coverslips were fixed, permeabilized, and incubated with primary antibodies against E-cadherin (Transduction Laboratories) or HECD-1 (Zymed Laboratories), and polyclonal anti–β-catenin antibody (Sigma Israel Chemicals Ltd.), at RT, as described previously (Sadot et al., 1998). The secondary antibodies were Alexa 488–conjugated goat anti–mouse or anti–rabbit IgG (Molecular Probes) and Cy3 goat anti–mouse or anti–rabbit IgG (Jackson ImmunoResearch Laboratories). Images were acquired using the DeltaVision system (Applied Precision) equipped with a microscope (model Axiovert 100; Carl Zeiss MicroImaging, Inc.) and Photometrics 300 series scientific-grade cooled CCD camera, reading 12-bit images, and using the 63×/1.4 NA plan-Neofluar objective. Adjustments of brightness, contrast, color balance, and final size of images was processed using Adobe Photoshop 5.5. Images of live cells (Fig. 6 A) were acquired with a 10×/0.25 NA lens using an invertoscope (model IM; Carl Zeiss MicroImaging, Inc.).

Western blotting

The antibodies used were described in the previous paragraph, and antibodies to tubulin, ERK, and P-ERK were from Sigma Israel Chemicals Ltd. Anti–ErbB-1 (sc-03) and P-ErbB-1 (sc-12351) were a gift from Y Yarden (Weizmann Institute of Science); ErbB-2 (sc-284) and anti-Slug antibodies were from Santa Cruz Biotechnology, Inc. Western blots were developed using the ECL method (Amersham Biosciences).

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