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Abstract

Transcriptional repression of *E-cadherin* is a characteristic feature of epithelial to mesenchymal transition (EMT) often found also during tumor cell metastasis. At the metastatic sites, the migratory fibroblastic cells sometimes revert to an epithelial phenotype. During colon cancer metastasis this process involves regulation of the E-cadherin-β-catenin complex. To investigate the molecular basis of this regulation, we used human colon cancer cells expressing aberrantly activated β-catenin signaling. Sparse cultures, mimicking invasive tumor cells, displayed very low levels of E-cadherin due to transcriptional repression of *E-cadherin* by Slug. Slug was induced by β-catenin signaling and, independently, by activated ERK. Dense cultures of colon cancer cells resembled a more differentiated epithelium displaying high levels of E-cadherin and β-catenin localized at cell-cell junctions. In such cells, β-catenin signaling, ErbB-1 and ErbB-2 levels, and ERK activation were reduced and Slug expression was undetectable. Inhibition of E-cadherin-mediated contacts in dense cultures, by anti E-cadherin antibody, resulted in nuclear localization and signaling by β-catenin, induction of Slug and inhibition of E-cadherin transcription, but without changes in ErbB-1 and ErbB-2 levels, and ERK activation. This autoregulation of E-cadherin expression mediated by cell-cell adhesion, involving the regulation of Slug by β-catenin and ERK, could be important in colon cancer development.

Introduction

Disruption of E-cadherin-mediated adhesion is considered a key step in progression towards the invasive phase of carcinoma (Behrens et al., 1992; Christofori and Semb, 1999; Takeichi, 1993). 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). In many types of cancer however, E-cadherin expression is lost without mutations in the gene (reviewed by Hirohashi, 1998) owing to transcriptional repression of *E-cadherin* (Batlle et al., 2000; Cano et al., 2000; Comijn et al., 2001; Hajra et al., 2002; Poser et al., 2001; Yokoyama et al., 2001). 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; Bolos et al., 2003; Cano et al., 2000; Comijn et al., 2001; Grooteclaes and Frisch, 2000; Nieto, 2002; Perez-Moreno et al., 2001).

Transcriptional repression of *E-cadherin* and the associated morphological changes in cells also occur during embryonic development in the course of epithelial to mesenchymal transition (EMT), when epithelial cells move into new microenvironments and differentiate into various cell types, for example during neural crest cell migration from the neuroectoderm (reviewed by Savagner, 2001; Thiery, 2002). Some of these processes, involving changes in E-cadherin, were also shown to involve the activation of β-catenin signaling (Eger et al., 2000; Logan et al., 1999; 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 signal by 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 also maintains the proliferative compartment in the 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; Conacci-Sorrell et al., 2002a; Polakis, 2000). 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 N-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) 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 the acquisition of invasive and metastatic capacities by the tumor cells, require additional 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), 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 downregulation of E-cadherin and nuclear localization of β-catenin at the invasive front, followed by re-formation 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 study 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: one, involving transcriptional activation of *Slug* by the β-catenin-Tcf complex and the other, activation of the ERK pathway. When adherens junctions are established in dense cultures, ErbB-1, ErbB-2 and the ERK pathway becomes

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 demonstrate the 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

SW480 human colon carcinoma cell cultures grown in sparse culture ($8x10^3$ cells/cm^2) for 2 days were characterized by weak and diffuse staining of E-cadherin, but strong nuclear β-catenin localization (Fig. 1A, sparse). In contrast, cells grown for 2 days as dense cultures ($6x10^4$ cells/cm^2) displayed a much stronger E-cadherin and β-catenin staining confined to cell-cell contacts, and a dramatic reduction in nuclear β-catenin (Fig. 1A, dense). This re-localization of β-catenin from the nucleus to adherens junctions in dense cultures was associated with a decrease in β-catenin-mediated signaling as demonstrated by the reduction in activation of a transfected, β-catenin-LEF/TCF-responsive reporter plasmid (Fig. 1B). 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 of cells, β-catenin signaling was maximal (Fig. 1B, lanes 5, 6), while with increasing culture density β-catenin signaling was dramatically reduced (Fig. 1B, lanes 1, 2). Dense cell cultures also showed a major increase in E-cadherin levels and its accumulation in the Triton X-100-insoluble membrane-cytoskeleton fraction (Fig. 1C, lane 2, compare to lanes 4 and 6). Two other colon cancer cell lines, HCT116 and SW48 that express β-catenin with stabilizing mutations in its N-terminus, also displayed an increase in E-cadherin level in dense compared to sparse cultures (Fig. 1D). The difference between sparse and dense cultures of these two cell lines was less dramatic (see below Fig. 3) since they also express significant levels of E-cadherin in sparse cultures, unlike SW480 cells (Cano et al., 2000; Gottardi et al., 2001). MDCK and MDBK epithelial cells expressing wt β-catenin, did not display such density dependent regulation of E-cadherin expression (data not shown).

Downregulation 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)-containing RNA from SW480 cells cultured for 2 days at different densities (Fig. 2A, upper panel). A significant elevation in E-cadherin mRNA content occurred with increasing culture density

(Fig. 2A upper panel, lanes 3, 4 compare to lane 1). Moreover, the transcriptional activity of a reporter plasmid containing the *E-cadherin* promoter was 5 to7 fold higher in dense cultures than in sparse cell cultures (Fig. 2D lane 1 compare to lane 2). Since 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 for Snail and Slug in these SW480 cultures. Snail mRNA levels did not change significantly between sparse and dense cultures (Fig. 2A third panel), but the level of Slug mRNA was very high in sparse cultures (Fig. 2A second panel, lane 1), but undetectable in more dense cultures (Fig. 2A second panel, lanes 2-4), suggesting that Slug is involved in transcriptional repression of *E-cadherin* in sparse SW480 cells. The expression of Slug was rapidly induced after seeding the cells in sparse culture, peaking between 3-6 hours after seeding (Fig. 2B lanes 1-6), while in dense cultures the low level of Slug induced between 3-6 hours was lost at later times when the cells established cell-cell contacts (Fig. 2B, lanes 7-12). We also determined the kinetics of the *E-cadherin* promoter response to culture density by transfecting an *E-cadherin* reporter plasmid into dense cultures, followed by trypsinization of the cells after 14 hours, and seeding the cells as sparse and dense cultures. E-cadherin promoter activity decreased significantly at early times in sparse cultures, but not in dense cultures (Fig. 2C, 12 hours), in agreement with the early increases in Slug in sparse cultures (Fig. 2B). At later times, when cells established contacts, *E-cadherin* promoter activity increased (Fig. 2C, 28 hours).

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

dense cells where E-cadherin expression was high (Fig. 3D), as observed in SW480 cells (Fig. 2A).

To confirm that Slug can repress *E-cadherin* transcription in SW480 cells, the Slug cDNA was co-transfected with the wt *E-cadherin* reporter, or with a mutant E-box *E-cadherin* promoter into sparse and dense cultures of SW480 cells. Slug was very efficient in repressing the activity of wt *E-cadherin* promoter in dense cultures (Fig. 2D lane 3, compare to lane 1), but could only weakly affect the mutant E-box promoter (Fig. 2D lane 7 compare to lane 5). The E-box mutant was more active in sparse cultures than the wt promoter (Fig. 2D lane 6 compare to lane 2), most probably since endogenous Slug could not bind and inhibit its activity. Moreover, transfected Slug was unable to suppress the mutant E-box promoter (Fig. 2D lane 8, compare to lane 6). Since the E-box mutant was still regulated (albeit weakly) by cell density (Fig. 2D compare lane 6 to 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. 2E lanes 2 and 4 compare to lanes 1 and 3, respectively).

To directly test whether Slug can affect the endogenous E-cadherin level 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 a dramatic reduction in E-cadherin levels (Fig. 2F upper panel) that changed the morphology of the transfected cells to an extended fibroblastic shape. In contrast, the neighboring untransfected cells had an epithelial shape. Transfection of GFP alone had no effect on cell morphology or E-cadherin levels (Fig. 2F lower panel).

Slug is activated by β-catenin/TCF signaling

Since Slug mRNA levels were high in sparse cultures of SW480 cells displaying nuclear β-catenin and strong β-catenin-mediated transactivation, while dense cultures lacked Slug and had low β-catenin signaling capacity (Figs. 1-3), we tested if the high Slug levels in sparse cultures can result from activation of *Slug* by β-catenin signaling. Co-transfection of a mouse *Slug* promoter reporter together with β-catenin into 293-T cells showed that the *Slug*

promoter is activated by transfected β-catenin (Fig. 4A lane 2 compare to lane 1), and also by endogenous β-catenin in SW480 cells (Fig. 4B lane 1). In contrast, *Snail* promoter activity was not induced by β-catenin (Fig. 4A lanes 5 and 6). In 293 cells, transient transfection of GFP-tagged Slug very effectively reducing endogenous E-cadherin levels (Fig. 4C lane 3 compare to lane 1), while transfection of GFP-Snail had only a mild effect (Fig. 4C lane 2 compare to lane 1). Dominant negative Tcf blocked activation of the *Slug* promoter (Fig. 4A lane 3 compare to lane 2, and Fig. 4B lane 2 compare to lane 1), similar to the cytoplasmic domain of cadherin that sequesters β-catenin from binding to Tcf (Fig. 4A lane 4 compare to lane 2 and Fig. 4B lane 3 to 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 cytoplasmic domain of cadherin (Shtutman et al., 1999) and displaying decreased β-catenin-dependent transactivation (Fig. 4D). Clones expressing high levels of the cadherin tail (SW480-7 and SW480-8), had more E-cadherin than control cells, or clone SW480-6 cells, expressing very low levels of the cadherin tail (Fig. 4E lanes 7 and 8 compare to lanes 5 and 6). In agreement with the changes in E-cadherin protein level the E-cadherin RNA (Fig. 4F) levels were also higher in clones SW480-7 and SW480-8 than in control SW480 cells. This was especially evident in sparse cultures (Fig. 4E lanes 5 and 6 compare to lane 4). Inhibition of β-catenin signaling resulted in decreased Slug levels in SW480-7 and SW480-8 cells compared to parental SW480 cells (Fig. 4G lanes 3 and 4 compare to lane 2). Transcriptional activity of the *Slug* promoter in SW480-8 cells was also lower than in control cells (Fig. 4H).

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

ERK activation regulates E-cadherin and Slug expression

Since the regulation of E-cadherin 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 expression in SW480 cells involves ERK activation. We found very high levels of activated ERK in sparse, compared to dense cultures (Fig. 5A third panel, compare lanes 1 and 3). Inhibition of the ERK pathway by PD98059 (Fig. 5A third panel, lane 2 compare to lane 1) induced an increase in E-cadherin levels of sparse cultures (Fig. 5A upper panel, lane 2 compare to lane 1), but had no effect in dense cultures that had no detectable activated ERK (Fig. 5A first second and third panels lanes 3 and 4). An increase in E-cadherin RNA level was also observed in the presence of PD98059, especially in sparse cell cultures (Fig. 5B lanes 3 and 4). We also tested the ability of ERK to affect *E-cadherin* promoter activation and found that PD98059 enhanced *E-cadherin* promoter activity in sparse cultures (Fig. 5C lane 2 compare to lane 1), but had a weaker effect in dense cultures (Fig. 5C lane 8 compare to lane 7). Transfection of constitutively active ERK (MEK1SSDD) inhibited *E-cadherin* promoter activity in dense cultures (Fig. 5C lane 9 compare to lane 7), but had no effect in sparse cultures (Fig. 5C lane 3 compare to lane 1), since these cells already displayed high levels of activated endogenous ERK (Fig. 5A third panel, lane 1). Interestingly, MEK1SSDD did not affect the E-box mutant *E-cadherin* promoter (Fig. 5C lane 6 compare to lane 4), which was very active also in sparse cultures (Fig. 2D lane 6 compare to lane 2), indicating that activated ERK regulates the *E-cadherin* promoter by also involving the E-box domain. To ask if ERK affects *E-cadherin* transcription by inducing Slug expression, sparse cell cultures were treated with two different inhibitors of the ERK pathway (PD98059 and UO126), and both were found to reduce the level of Slug (Fig. 5D lanes 2 and 3, compare to 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.

Since induction of the ERK pathway usually results from activation of tyrosine kinase growth factor receptors, we examined whether their inhibition by tyrphostin AG1478 elevates E-cadherin levels in sparse cultures displaying activated ERK. AG1478 was most effective in elevating E-cadherin expression in sparse cells (Fig. 5E lane 6 compare to lane 5) and

semi-confluent cultures (Fig. 5E lane 4 compare to lane 3), where it reduced ERK activation, but not in dense cultures lacking activated ERK (Fig. 5E 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 cultures, but very low in dense cultures (Fig. 5F). We also examined whether soluble factors secreted by sparse cultures are involved in stimulating tyrosine kinase receptors, 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 of sparse and dense cultures was exchanged. We did not observe in such experiments any changes in E-cadherin expression either in sparse or dense cultures (data not shown).

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 expression are 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. 5G). Moreover, in clones expressing the cadherin tail (and therefore having reduced β-catenin signaling), inhibition of ERK by PD98059 or UO126 elevated E-cadherin expression to an extent similar to that observed in control cells (Fig. 5H lanes 2, 3; 5, 6; and 8, 9, compare to lanes 1, 4 and 7, respectively). This suggests that when β-catenin signaling is inhibited, blocking ERK can still elevate E-cadherin levels, indicating that ERK activation and β-catenin signaling can independently repress E-cadherin expression.

Cadherin-cadherin interactions enhance E-cadherin expression

We also determined in dense cultures of SW480 cells if the assembly of adherens junctions is involved in the induction of E-cadherin expression. To inhibit E-cadherin-dependent adherens junctions assembly in long term cultures (48 hours), dense cultures were seeded in the presence of a polyclonal antibody against the extracellular domain of E-cadherin to block cadherin-cadherin interactions. Cells cultured with this antibody had an altered colony

morphology with scattered cells, compared to cells cultured in the presence of control antibody that were organized in colonies (Fig. 6A,b compare to 6A,a). In the presence of the antibody the organization of β-catenin underwent a dramatic change, opposite to that described in Fig. 1A: instead of localizing to cell-cell contact areas (Fig. 6A, c), β-catenin re-localized to the nuclei of cells with only little β-catenin found in adherens junctions (Fig. 6A, d). Cells that were first transfected with the TOPFLASH reporter, 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 to control (Fig. 6B and C lanes 2, compare to lanes 1, respectively). Expression of Slug was higher in dense cultures incubated with the antibody (Fig. 6D lane 3 compare to lane 2), but was significantly lower than in sparse cultures (Fig. 6D lane 3 compare to lane 1). Expression of E-cadherin RNA and protein in cells incubated with anti E-cadherin antibody was also reduced compared to control cultures (Fig. 6E lanes 2 and 3 compare to lane 1; Fig. 6F lanes 3, 4 compare to lanes 1, 2). The inhibition in E-cadherin expression did not involve the induction of ErbB-1 and ErbB-2 expression or activity (Fig. 6D panels 2-4, lanes 2 and 3, compare to lanes 1, respectively) or ERK activity (Fig. 6F, second panel) 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 both required for full regulation, by a positive feedback mechanism driven by growth factor receptors and the assembly of adherens junctions.

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 (reviewed in 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 study, 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), while the cells regained an epithelial phenotype from a more fibroblastic one. The sparse cultures of SW480 and HCT116 cells more closely resembled the cells at the invasive front of colon carcinoma cells, 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 exerted by two different pathways (Fig. 7): One, involving activated receptor tyrosine kinases 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 (V. Arnoux, C. Come, and P. Savagner, manuscript in preparation), inhibition of *Slug* promoter activation by dominant negative Tcf (Fig. 4A, 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, an induction of Slug. Moreover, transfection of Slug into SW480 cells abolished E-cadherin expression (Fig. 2F) and there was a correlation in the kinetics of Slug induction in sparse cell cultures (Fig. 2B) and transcriptional repression of the *E-cadherin* promoter (Fig. 2C). When ErbB-1 and ErbB-2 levels and activities were reduced (and ERK signaling inhibited), the activity of wt *E-cadherin* promoter was elevated, while that of an E-box mutant was not (Fig. 5C). 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. Since 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. 2B), Slug was most probably responsible for downregulating *E-cadherin* transcription in sparse cultures of SW480 cells. This view is supported by our finding that the *Slug*, but no the *Snail*, promoter was activated by β-catenin (Fig. 4A) and Slug transfection was effective, while that of Snail was not, in reducing endogenous E-cadherin in 293 cells (Fig. 4C).

Interestingly, inhibition of the integrin-linked kinase (ILK) 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). Since β-catenin signaling is not involved in *Snail* promoter regulation (Fig. 4A), the mechanism(s) involved in Snail regulation by ILK in colon cancer cells are yet unknown.

Previous studies suggested a link between increased β-catenin signaling and downregulation of E-cadherin expression 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 downregulation of E-cadherin (Weng et al., 2002). These studies support our observation that β-catenin-Tcf signaling and ERK activation repress the expression of E-cadherin in sparse SW480 colon cancer cells. We have shown, in addition, that this regulation operates

by the induction of Slug. While the induction of β-catenin leads to ERK activation in RK3E cells, in SW480 colon cancer cells ERK activation and β-catenin signaling could operate independently of each other to induce the expression of Slug (Figs. 5-7). This difference may have resulted from the difference between normal epithelial and carcinoma cells, or from the use of transfected β-catenin chimera in RK3E cells, and 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 in 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 display activated ERK and did not express Slug, thus relieving the repression on E-cadherin transcription and allowing E-cadherin expression. An association between cell culture density and MAPK (ERK) activity could reflect both in vitro and in vivo a modulation in tyrosine kinase receptor activity or expression of the ErbB-1/ErB-2 family (Fig. 5F), as also described for other cultured carcinoma cells (Savagner et al., 1997; Takahashi and Suzuki, 1996).

The increase in E-cadherin expression in dense cultures resulted in re-localization 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 also displayed a lower percentage of cells in S-phase of the cell cycle compared to sparse cultures (data not shown), in agreement with recent studies showing 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 study also showed that disruption of E-cadherin-mediated cell-cell adhesion, by an antibody to E-cadherin, induces the proliferation of 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 expressing differentiated tubular colon carcinoma cells and the diminished level of such markers in the invasive colon cancer cells displaying nuclear β-catenin (Brabletz et al., 2001). These discrepancies most probably

result from the different microenvironment around tumor cells in vivo as compared to cells cultured in vitro.

In dense cultures, the increased expression of E-cadherin could be reduced when the cells were grown in the presence of anti E-cadherin antibody (Fig. 6E and F) that inhibited cadherin-cadherin interactions, and Slug was rapidly induced in sparse cultures following dense cell culture dispersion after trypsinization (Fig. 2B). The re-localization of β-catenin to nuclei and induction of its signaling activity and of Slug expression (albeit partial) (Fig. 6D lanes 2 and 3 compare to lane1), 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 its ability to function (at least in part) independently of the ERK pathway.

β-catenin-Tcf signaling is required in the intestinal epithelium in the proliferative compartment at the bottom of the 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 compartment 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 the very sparse colon cancer cell cultures (Fig. 2A, B; 3A, D) that display the highest level of β-catenin signaling (Fig. 1B; 3B) and lack adherens junctions (Fig. 1A), similar to cells at the invasive front of colon tumors (Brabletz et al., 2001). Such very 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 study showed that such inverse relationship between β-catenin nuclear localization and signaling and downregulation of E-cadherin expression, is also an integral part of hair follicular bud development (Jamora et al., 2003) and 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 receptor tyrosine kinases and downstream components of the MAPK pathway, and the conditions responsible for triggering *Slug* repression when cells establish contacts and acquire an epithelial phenotype.

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Materials and methods

Cell lines, cell culture and transfections

SW480, 293-T and HCT116 cells were grown in DMEM with 10% bovine calf serum. SW480 cells expressing the N-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 semiconfluent culture was seeded into 35 mm dishes as dense ($6x10^4$; $3x10^4$ cells/cm^2), medium ($2x10^4$; $1.5x10^4$ cells/cm^2) and sparse ($8x10^3$; $6x10^3$ cells/cm^2) cultures, from one original dish. After 48 hours, 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 tyrosine kinase receptor inhibitor AG1478 (500 nM) (all from Calbiochem, San Diego, CA), for the last 24 hours before cell harvesting. To disrupt adherens junctions, SW480 cells were incubated for 48 hours with 1:10 or 1:50 dilutions of polyclonal antibody against the extracellular domain of human E-cadherin, kindly provided by Dr. M. Wheelock (Univ. Omaha, Nebraska). Transient transfections into SW480 and 293-T cells were carried out with lipofectamin (Gibco-BRL). For transactivation assays, 0.5 μg β-galactosidase plasmid was co-transfected with 1 μg of reporter plasmids and 3.5 μg β-catenin S33Y, or the Slug construct, in duplicate plates, and after 48 hours luciferase and β-galactosidase activities were determined as described (Shtutman et al., 1999).

Plasmids

The wt and E-box mutant mouse *E-cadherin* promoters provided by Dr. 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 promoter was cloned from a mouse embryonic library prepared by Y. Yamada (NIH, Bethesda, MD) using a mouse cDNA probe (Savagner et al., 1997). The promoter region was sequenced (V. Arnoux, C. Come and P. Savagner, manuscript in preparation) and cloned into the BglII-KpnI sites of pGL3 fused with luciferase. The human

E-cadherin and *Snail* promoters cloned into pGL3 were from Dr. A. G. de Hereros (Universitat Pompeu Fabra, Barcelona, Spain). TOPFLASH, FOPFLASH, and dominant negative TCF4 (ΔNTCF4) were provided by Drs. H. Clevers and M. van de Wetering (Univ. Med. Center, Utrecht, The Netherlands). Human Slug, a gift from T. Ip (U. Mass Med. Sch., Worcester, MA) and Snail cDNAs were cloned into the pTracer expression vector encoding for GFP under the control of an independent promoter (F. Magnino, F. Bibeau, C. Come, C. Theillet, and P. Savagner, manuscript in preparation). 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 (Sadot et al., 1998). The MEK1SSDD plasmid was provided by Dr. J. Pouyssegur (Inst. Signaling, Dev. Biol. and Cancer Res., Nice, France) and Dr. B. Boyer (Institute Curie, Paris-Sud, France).

RNA analysis

Northern blot hybridization was carried out 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 Dr. J. Behrens (Max-Delbruck-Center Mol. Med., Berlin, Germany), ^{32}P-labeled mouse Snail cDNA, from Dr. 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 (Batlle et al., 2000).

Immunofluorescence

Cells cultured on glass coverslips were fixed, permeabilized and incubated with primary antibodies against E-cadherin (Transduction Laboratories) and polyclonal anti-β-catenin antibody (Sigma-Israel, Nes Ziona, Israel) as described (Sadot et al., 1998). The secondary antibodies were Alexa 488 conjugated goat anti-mouse or anti-rabbit IgG (Molecular Probes, Leiden, The Netherlands) and Cy3 goat anti-mouse or anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA).

Western blotting

The antibodies used were described above, and antibodies to tubulin, ERK and P-ERK were from Sigma Israel (Nes Ziona, Israel). Anti ErbB-1 (sc-03), P-ErbB-1 (sc-12351), ErbB-2 (sc-284) and anti Slug antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Western blots were developed using the ECL method (Amersham, Buckinghampshire, UK).

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Figure legends

Figure 1. **Regulation of E-cadherin expression and β-catenin localization and signaling by cell density**. (A) SW480 cells were seeded from a semiconfluent culture dish at $6x10^3$ cells/cm^2 (sparse) and $6x10^4$ cells/cm^2 (dense), and after 2 days double stained for E-cadherin and β-catenin. (B) Cells cultured at different densities: lane 1, $6x10^4$ cells/cm^2; lane 2, $3x10^4$ cells/cm^2; lane 3, $2x10^4$ cells/cm^2; lane 4, $1.5x10^4$ cells/cm^2; lane 5, $1x10^4$ cells/cm^2; lane 6, $8x10^3$ cells/cm^2, (S, sparse; M, medium; D, dense), were transiently transfected with TOPFLASH (TOP) or FOPFLASH (FOP) reporters and fold activation was determined in duplicate dishes. (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 and SW48 colon cancer cells were grown as sparse ($6x10^3$ cells/cm^2) and dense ($6x10^4$ cells/cm^2) cultures and the level of E-cadherin and tubulin was determined. Note induction of E-cadherin expression, re-localization of β-catenin to the membrane and inhibition of β-catenin-mediated transactivation in dense cultures. The bar in (A) is 10 μm.

Figure 2. **Inhibition of E-cadherin expression in sparse cultures by Slug.** (A) SW480 cells were seeded at: lane 1, $6x10^3$ cells/cm^2, lane 2, $2x10^4$ cells/cm^2, lane 3, $4x10^4$ cells/cm^2, and lane 4, $8x10^4$ 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. 1A), 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 hours 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. (F)

SW480 cells were transfected with a plasmid coding for both GFP and Slug, or GFP alone, and stained for E-cadherin with rhodamine labeled secondary antibody. Note that Slug expression correlated with low levels of E-cadherin, it inhibited the wt E-cadherin promoter and reduced E-cadherin expression in transfected cells. The bar in (F) is 10 μm.

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 (see Fig. 1) and after 48 hours 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. (D) The levels of E-cadherin and Slug were determined by western blot analysis 30 hours after cell seeding at the densities indicated in Fig. 1A. The bar in (A) represents 10 μm.

Figure 4. **Activation of *Slug* transcription by β-catenin-TCF signaling.** (A, B) Activation of mouse *Slug*, but not Snail, promoter by β-catenin in 293-T (A) and SW480 cells (B), and inhibition of this activation by dominant negative TCF (ΔNTCF4), or the cytoplasmic tail of cadherin (Cad tail). (C) Transfection of Slug, but not 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. (D) Decreased β-catenin/TCF-mediated transactivation in SW480 clones (SW480-7 and SW480-8) stably expressing different levels of the cadherin tail. SW480 clones expressing the cadherin tail displayed elevated E-cadherin expression, (E) increased E-cadherin protein and (F) RNA, but (G) decreased Slug protein, and (H) lower Slug promoter activity.

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 hours 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. (D) Inhibition of ERK activation by PD98059 and UO126 in sparse cultures reduced the level of Slug. (E) The tyrosine kinase receptor 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 to control SW480 cells and, (H) inhibition of ERK activation in these cells by PD98059 and UO126 elevated E-cadherin levels.

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. (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. The bar in (A, d) is 10 μm and 50 μm in (A, b).

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 involving activation of ERK (P-ERK) by tyrosine kinase receptors (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*. Another 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.