

# E-cadherin controls $\beta$ -catenin and NF- $\kappa$ B transcriptional activity in mesenchymal gene expression

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## Summary

E-cadherin and its transcriptional repressor Snail1 (*Snai1*) are two factors that control epithelial phenotype. Expression of *Snai1* promotes the conversion of epithelial cells to mesenchymal cells, and occurs concomitantly with the downregulation of E-cadherin and the upregulation of expression of mesenchymal genes such as those encoding fibronectin and LEF1. We studied the molecular mechanism controlling the expression of these genes in mesenchymal cells. Forced expression of E-cadherin strongly downregulated fibronectin and *LEF1* RNA levels, indicating that E-cadherin-sensitive factors are involved in the transcription of these genes. E-cadherin overexpression decreased the transcriptional activity of the fibronectin promoter and reduced the interaction of  $\beta$ -catenin and NF- $\kappa$ B with this promoter. Similar to  $\beta$ -catenin, NF- $\kappa$ B was found, by co-immunoprecipitation and pull-down assays, to be associated with E-cadherin and other cell-adhesion components. Interaction of the NF- $\kappa$ B p65 subunit with E-

cadherin or  $\beta$ -catenin was reduced when adherens junctions were disrupted by K-ras overexpression or by E-cadherin depletion using siRNA. These conditions did not affect the association of p65 with the NF- $\kappa$ B inhibitor I $\kappa$ B $\alpha$ . The functional significance of these results was stressed by the stimulation of NF- $\kappa$ B transcriptional activity, both basal and TNF- $\alpha$ -stimulated, induced by an E-cadherin siRNA. Therefore, these results demonstrate that E-cadherin not only controls the transcriptional activity of  $\beta$ -catenin but also that of NF- $\kappa$ B. They indicate too that binding of this latter factor to the adherens junctional complex prevents the transcription of mesenchymal genes.

Supplementary material available online at <http://jcs.biologists.org/cgi/content/full/121/13/2224/DC1>

Key words: Cadherin, EMT, NF- $\kappa$ B, Snail,  $\beta$ -catenin

## Introduction

Epithelial cells have a high degree of plasticity: they can convert to a mesenchymal phenotype in a process known as epithelial-to-mesenchymal transition (EMT) (Savagner, 2001; Huber et al., 2005). This phenomenon, essential for embryo development, is also required for the acquisition of invasive properties by cancer cells. EMT is also reversible, and conversion of mesenchymal to epithelial cells (MET) happens during some processes of organogenesis and in micrometastasis (Thiery, 2002). Because expression of E-cadherin protein is the classic feature of epithelial cells, E-cadherin is downregulated during EMT and upregulated in MET. A transcriptional consequence of the presence of E-cadherin in epithelial cells can be inferred from the normal association of E-cadherin with  $\beta$ -catenin in adherens junctions. This prevents  $\beta$ -catenin transfer to the nucleus and impedes its role as a transcriptional activator, which occurs through its interaction mainly with the TCF (T-cell factor)-LEF (lymphoid enhancer factor) family of transcription factors, but also with other DNA-binding

proteins (Gordon and Nusse, 2006). Accordingly, the involvement of  $\beta$ -catenin signalling in EMTs during tumour invasion has been established (Brabletz et al., 2005).

Evidence from several laboratories indicates that the E-cadherin repressor Snail1 (*Snai1*) is essential for triggering EMT (Huber et al., 2005; Barrallo-Gimeno and Nieto, 2005; De Craene et al., 2005). Overexpression of Snail1 induces the repression of E-cadherin and other epithelial markers, and the activation of mesenchymal markers such as fibronectin or LEF1 (Batlle et al., 2000; Cano et al., 2000; Guaita et al., 2002). Repression of E-cadherin and other epithelial genes is caused by the direct interaction of the Snail1 C-terminal domain with specific 5'-CACCTG-3' core sequences present in the promoters (Batlle et al., 2000; Cano et al., 2000). However, the mechanisms leading to the activation of mesenchymal genes are not yet known. Here, we investigated the molecular pathways controlling the expression of fibronectin and LEF1. Our results indicate that E-cadherin prevents the transcription of these genes and the binding not only of  $\beta$ -catenin but also of NF- $\kappa$ B, another

transcriptional factor associated with EMT, to their promoters (Huber et al., 2004). Moreover, similar to  $\beta$ -catenin, NF- $\kappa$ B was found to be associated with E-cadherin complexes in epithelial cells.

## Results

### E-cadherin interferes with the induction of fibronectin and *LEF1* transcription

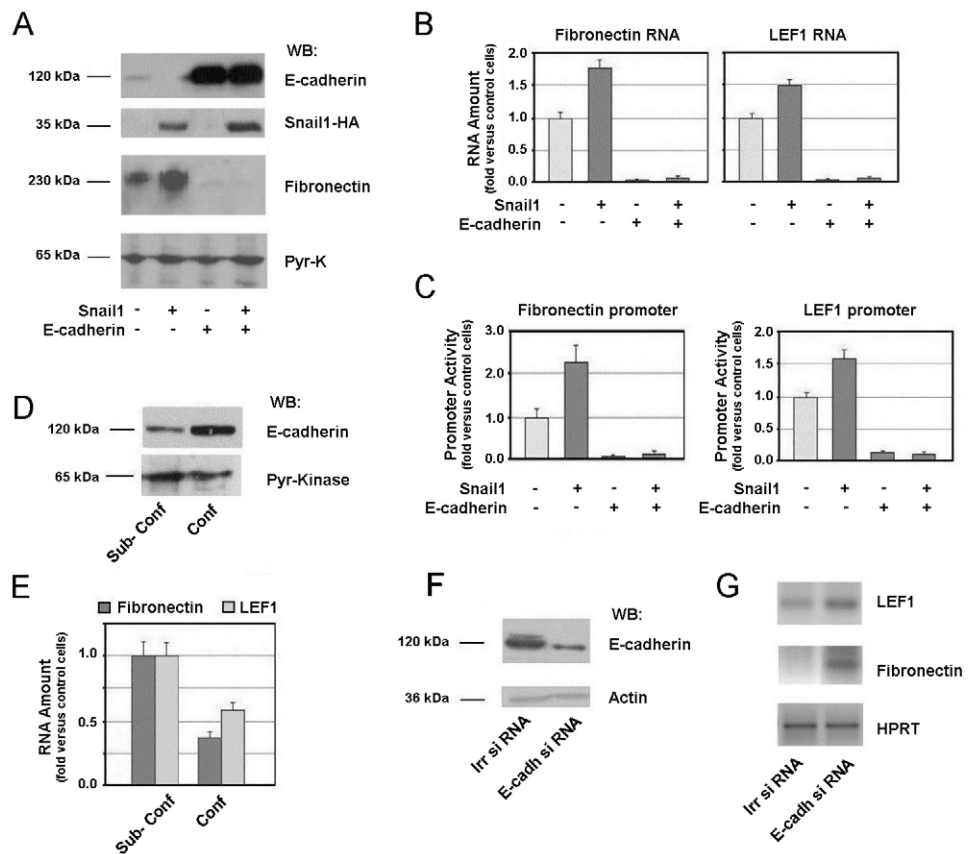
Increased expression of mesenchymal markers has been detected in epithelial cells that have undergone EMT. This is the case of fibronectin and *LEF1* mRNAs, which are upregulated during Snail1-induced EMT in HT-29 M6 epithelial cells (Guaita et al., 2002) (supplementary material Fig. S1). Activation of these genes by Snail1 was also reproduced in other intestinal epithelial cell lines, such as LS174T (see below) and to a lesser extent in SW-480 cells (Fig. 1A,B), probably because SW-480 cells already express these markers when grown at low confluence. Downregulation of ectopic Snail1 expression in inducible HT-29 M6 Snail1 transfectants was found to restore E-cadherin mRNA levels at the same time as it downmodulates fibronectin and *LEF1* RNAs (supplementary material Fig. S1). Therefore, we checked whether E-cadherin modulates the expression of these two genes. SW-480 cells were transfected with E-cadherin under the control of a constitutive promoter and the RNA levels of fibronectin and *LEF1* were analysed. As shown in Fig. 1B, E-cadherin expression severely downregulated fibronectin and *LEF1* RNA levels;

normal levels could not be restored by Snail1 co-expression. Neither Snail1 nor E-cadherin expression induced significant changes in *HPRT* (hypoxanthine guanine phosphoribosyl transferase) RNA, used as control. Ectopic expression of E-cadherin in SW-480 cells also repressed the activity of fibronectin and *LEF1* promoters. Both a  $-341/+265$  fibronectin promoter and a  $-735/+1077$  *LEF1* promoter were potently downregulated in SW-480 cells expressing E-cadherin (Fig. 1C), in either the presence or absence of Snail1.

To confirm that the interference on mesenchymal gene expression by overexpressed E-cadherin is also reproduced by the endogenous E-cadherin, we cultured SW-480 ADH cells at higher cell density. This cell line is used for studying epithelial plasticity, because subconfluent sparse cultures mimic invasive tumour cells and show low levels of E-cadherin, whereas confluent cultures resemble a more differentiated epithelium with higher amounts of E-cadherin (Conacci-Sorell et al., 2003). As seen in Fig. 1D, the levels of E-cadherin were increased after confluence. This upregulation in endogenous E-cadherin levels was accompanied by a 50% decrease in fibronectin and *LEF1* RNAs (Fig. 1E).

E-cadherin levels were also modulated in another cell system. IEC-18 is an epithelial cell line with high E-cadherin expression and very low expression of fibronectin and *LEF1*. Transfection of this cell line with a specific small interfering RNA (siRNA) for E-cadherin reduced the levels of this protein (Fig. 1F). Concomitantly,

**Fig. 1.** E-cadherin represses fibronectin and *LEF1* gene expression. (A) Levels of E-cadherin and Snail1 proteins in SW-480 transfectants. SDS-protein extracts obtained from SW-480 cells stably transfected with the indicated genes and grown until 50–60% confluence were analysed by western blot (WB) with the indicated antibodies. (B) E-cadherin expression downregulates fibronectin and *LEF1* RNA levels. Fibronectin and *LEF1* RNAs were determined by qRT-PCR in SW-480 cell lines. Values are relative to that obtained in control SW-480 cells. Graphics show the average  $\pm$  s.d. of the three values obtained for every sample. (C) E-cadherin expression downregulates fibronectin and *LEF1* promoter activity. Activities of  $-341/+265$  fibronectin promoter and  $-735/+1077$  *LEF1* promoter were determined after transfection of these promoters, which were inserted into pGL3 plasmid as described, into subconfluent SW-480 stable transfectants. The values show the average  $\pm$  s.d. of two experiments performed in triplicate samples, and are relative to the value obtained in control SW-480 cells. (D) Cell-culture confluence regulates SW-480 E-cadherin levels. SW480 ADH cells were grown in standard conditions until 50–60% confluence (Sub-Conf) or 3 days after 100% confluence (Conf). 1% SDS total protein extracts were obtained and analysed by western blot with anti-E-cadherin or anti-pyruvate-kinase mAbs. (E) Expression of fibronectin and *LEF1* decrease in confluent SW-480 cells. Fibronectin and *LEF1* RNAs were determined by qRT-PCR and values (average  $\pm$  s.d.) referred to the value obtained in the sub-confluent cells. (F,G) Interference of E-cadherin expression upregulates fibronectin and *LEF1* RNA levels. Cells expressing an siRNA specific to E-cadherin or a scrambled control (Irr) were cultured until confluence, and E-cadherin and actin levels were determined by western blot (F). In parallel, fibronectin, *LEF1* or *HPRT* RNA content were determined in these cells by semi-quantitative RT-PCR (G).



fibronectin and *LEF1* RNAs were upregulated, as determined by semi-quantitative reverse transcriptase (RT)-PCR (Fig. 1G).

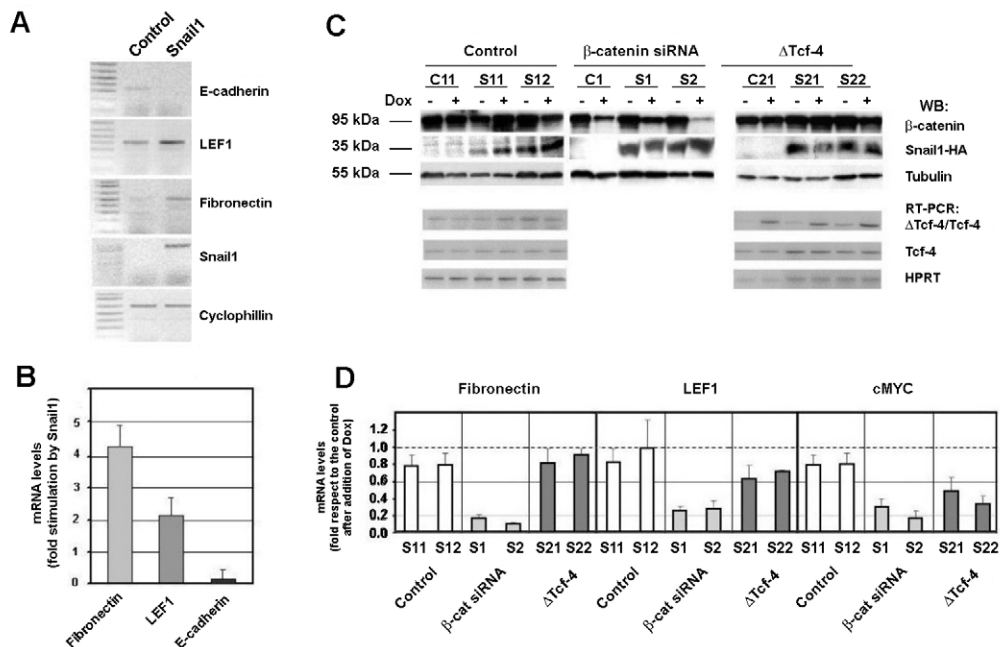
These results suggest that E-cadherin controls the expression of mesenchymal markers. Therefore, we investigated the molecular details of this negative control.

$\beta$ -catenin is required for the activation of mesenchymal genes. Our results suggest that an E-cadherin-dependent factor is required for transcription of these target genes. Therefore, we tested whether the E-cadherin-associated protein  $\beta$ -catenin was involved in the transcription of fibronectin and *LEF1*. Both the fibronectin and *LEF1* promoters contain putative binding sites for TCF4. However, whereas the *LEF1* promoter was stimulated by an activated form of TCF4 (VP16-TCF4) and inhibited by a negative mutant ( $\Delta$ TCF4), fibronectin was not (supplementary material Fig. S2), indicating that the TCF4-binding sites in the fibronectin promoter are not functional.

To check the relevance of  $\beta$ -catenin in fibronectin and *LEF1* gene expression, we used cell clones in which downregulation of  $\beta$ -catenin protein levels or of TCF4 transcriptional activity could be achieved. We took advantage of previously established LS174T cell clones, in which  $\Delta$ TCF4 mRNA or  $\beta$ -catenin siRNA are induced by doxycycline treatment (van de Wetering et al., 2002; van de Wetering et al., 2003). In LS174T cells, expression of *Snail1* increased the mRNA levels of *LEF1* and especially of fibronectin (Fig. 2A). As expected, E-cadherin mRNA was downregulated after expression of *Snail1* (Fig. 2A). Similar modulations of the

expression of these three genes were observed when *Snail1* was transfected either to control or to LS174T cells transfected with  $\Delta$ TCF4 or  $\beta$ -catenin siRNA in the absence of doxycycline. As shown in Fig. 2B, the increase in fibronectin RNA after *Snail1* transfection was higher than the increase of *LEF1*, which probably reflected the lower expression of fibronectin in control cells.

Treatment with doxycycline downregulated  $\beta$ -catenin protein levels only in clones treated with  $\beta$ -catenin siRNA, and not in control clones, which do not express this siRNA (Fig. 2C). The remaining  $\beta$ -catenin detected in these doxycycline-treated  $\beta$ -catenin siRNA clones was present at the cell membrane, as seen by immunofluorescence (supplementary material Fig. S3A). As expected,  $\Delta$ TCF4 mRNA increased specifically only in  $\Delta$ TCF4 mRNA clones. Because the primer set used for detecting  $\Delta$ TCF4 mRNA also amplified full-length endogenous *TCF4* mRNA, we performed a RT-PCR with a primer set amplifying only the full length. We found no changes in the level of endogenous *TCF4* mRNA in response to doxycycline, confirming a specific increase of the ectopic expression of  $\Delta$ TCF4 mRNA in these clones (Fig. 2C). In control clones, doxycycline did not induce changes in either  $\beta$ -catenin or  $\Delta$ TCF4 levels (Fig. 2C). The induction of both  $\beta$ -catenin siRNA and  $\Delta$ TCF4 repressed the activity of a  $\beta$ -catenin-sensitive promoter, TOP-Flash, by 75–80% in the different cell clones expressing *Snail1* (supplementary material Fig. S3B). Moreover, the expression of a well-characterised target of the  $\beta$ -catenin–TCF4 complex, *Myc*, was also affected by the expression of  $\beta$ -catenin siRNA or  $\Delta$ TCF4 (Fig. 2D), confirming that  $\beta$ -catenin



**Fig. 2.**  $\beta$ -catenin depletion downregulates fibronectin and *LEF1* transcript levels. (A,B) *Snail1* increases fibronectin and *LEF1* gene expression in LS174T cells. RNAs were extracted from LS174T control cells transfected with *Snail1* in a eukaryotic expression vector and analysed by semi-quantitative PCR (A) or qRT-PCR (B) with specific oligonucleotides for the indicated genes. Representative clones are shown in A; the average of the results obtained with three different clones are shown in B. (C) Inducible repression of  $\beta$ -catenin in LS-174T clones. Total-cell protein extracts or RNAs were obtained from clones expressing *Snail1* (clones S) or controls (clones C). Doxycycline (1  $\mu$ g/ml) was added for 6 days prior to the preparation of the extracts as indicated.  $\beta$ -catenin and *Snail1* expression were analysed by western blot with specific mAbs. Anti- $\alpha$ -tubulin was used as a loading control. Endogenous full-length *TCF4* mRNA and exogenous  $\Delta$ TCF4 plus endogenous *TCF4* mRNA were also analysed by RT-PCR. As a control, *HPRT* levels were determined. (D)  $\beta$ -catenin siRNA decreases fibronectin and *LEF1* transcript levels. RNA was obtained from the above-mentioned cell clones and levels of fibronectin, *LEF1* and *Myc* were determined by qRT-PCR. As a control, *HPRT* RNA levels were determined. The figure shows the values of fibronectin, *LEF1* and *Myc* RNA levels determined in the presence of doxycycline and referred to the level of the corresponding RNA in the absence of this drug. The average  $\pm$  s.d. of two independent experiments performed in duplicate with two representative clones is shown.

siRNA and  $\Delta TCF4$  were affecting the transcriptional activity of this complex.

We checked the requirement of  $\beta$ -catenin and TCF4 activity for the transcription of fibronectin and *LEF1*. In cells with expression of these genes, downregulation of  $\beta$ -catenin markedly decreased fibronectin RNA levels, whereas overexpression of  $\Delta TCF4$  did not (Fig. 2D). Doxycycline by itself did not interfere in these analyses, as shown by the small decrease detected in fibronectin RNA levels upon treatment of control cells with this drug (Fig. 2D). Therefore, the results indicate that a  $\beta$ -catenin-dependent and TCF4-independent mechanism mediates the transcription of the fibronectin gene. Similar results were obtained for *LEF1* RNA in  $\beta$ -catenin siRNA clones: *LEF1* RNA levels were decreased by doxycycline treatment in  $\beta$ -catenin siRNA clones, but not in control cells not expressing this siRNA (Fig. 2D, lane LEF1). Thus, a  $\beta$ -catenin-dependent signal controls the transcription of *LEF1*. A small decrease in *LEF1* levels was detected when  $\Delta TCF4$  was induced, suggesting that expression of this gene is also sensitive to a TCF4-dependent mechanism (Fig. 2D). A similar modulation of *LEF1* gene expression was observed in control LS174T cells, because, unlike fibronectin, these cells showed significant expression of *LEF1* (data not shown). Therefore, these results indicate that the expression of mesenchymal genes is under the control of  $\beta$ -catenin through TCF4-dependent or -independent complexes.

#### E-cadherin controls the association of $\beta$ -catenin with the fibronectin promoter

Because  $\beta$ -catenin was required for the expression of the mesenchymal genes, we checked whether the transcriptional activity of this protein was affected by ectopic E-cadherin. As shown in Fig. 3A, the activity of the  $\beta$ -catenin–TCF4-dependent promoter (TOP) was potentially downregulated by expression of E-cadherin in either SW-480 or SW-480 cells transfected with *Snail1*. We also analysed the association of  $\beta$ -catenin with the fibronectin promoter by chromatin immunoprecipitation (ChIP) assays. As shown in Fig. 3B,  $\beta$ -catenin was bound to the fibronectin proximal promoter. This association was increased (twofold) in SW-480 cells transfected with *Snail1* and totally downregulated in SW-480 cells overexpressing E-cadherin, in either the presence or absence of *Snail1*. This reproduced the changes detected in fibronectin RNA levels in these four conditions (see Fig. 1B). Binding of  $\beta$ -catenin to the fibronectin promoter correlated with the presence of  $\beta$ -catenin in the nucleus, as determined after cell fractionation (see below).

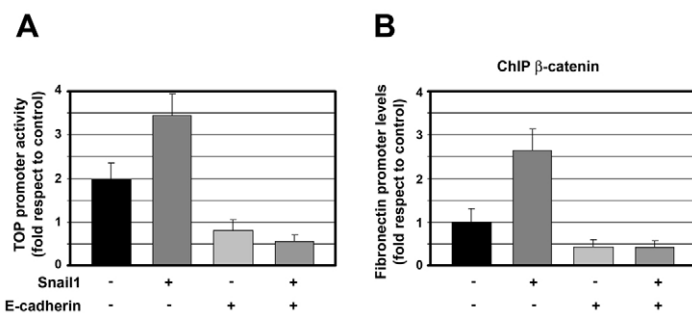
#### E-cadherin inhibits NF- $\kappa$ B transcriptional activity on the fibronectin promoter

Fibronectin gene expression is dependent on the activity of the transcriptional factor NF- $\kappa$ B (Chen et al., 2003). Indeed, in SW-

480 cells the activity of the fibronectin and *LEF1* promoters was upregulated by co-expression of VP16-Rel (supplementary material Fig. S4), a fusion chimera containing the Rel DNA-binding domain of NF- $\kappa$ B-p65 and the transactivator domain of VP-16. We checked with ChIP experiments whether binding of NF- $\kappa$ B to the fibronectin promoter was also modulated by *Snail1* and E-cadherin overexpression. Association of the p65 subunit of NF- $\kappa$ B to the fibronectin promoter was observed in SW-480 cells and was severely downregulated by E-cadherin expression, in the presence or absence of *Snail1* (Fig. 4A). The human fibronectin promoter contains a putative NF- $\kappa$ B-binding element placed between +35 and +48 (in relation to the transcription start site). Electrophoretic mobility shift assays (EMSA) performed with a probe corresponding to this sequence confirmed that p65 bound to this site. A specific retarded band was detected when we carried out the binding assays with nuclear extracts from SW-480 *Snail1*-transfected cells, and not with nuclear extracts from SW-480 cells transfected with *Snail1* and E-cadherin (Fig. 4B). This band was competed with an excess of an oligonucleotide corresponding to the consensus binding sequence for NF- $\kappa$ B, and not with a probe corresponding to a mutated version of this consensus binding element (Fig. 4B, left panel). Moreover, addition of a p65 antibody to the binding reaction prevented the formation of this complex, whereas an irrelevant IgG did not affect it (Fig. 4B, right panel). Therefore, we concluded that this band corresponded to a p65 complex.

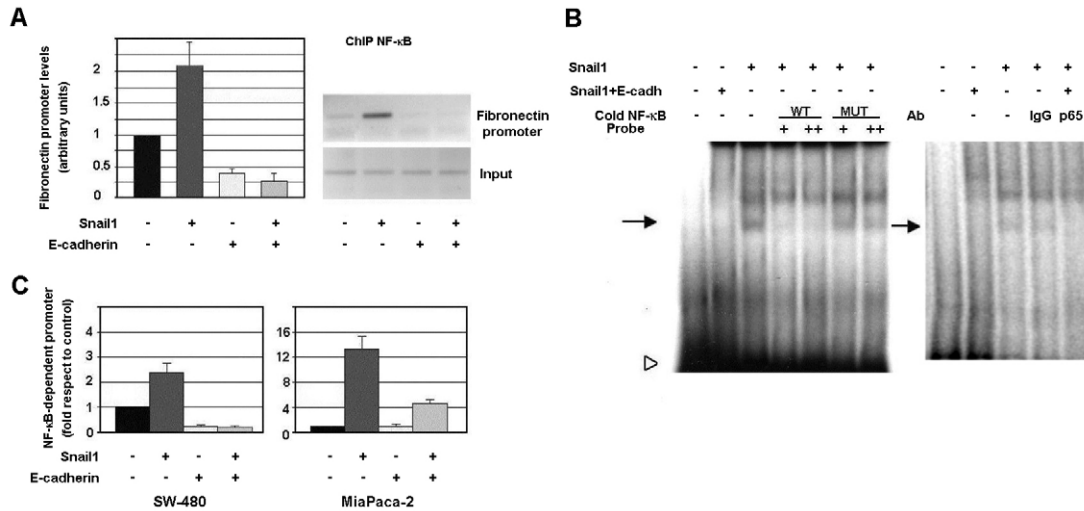
The activity of a synthetic NF- $\kappa$ B-sensitive promoter mirrored the results obtained with the mesenchymal gene promoters or with the TOP reporter plasmid: it was slightly (twofold) but consistently induced by *Snail1* expression and was totally repressed by E-cadherin (Fig. 4C). Similar results were obtained by transient transfection in another cell line: MiaPaca-2 cells deficient in E-cadherin expression. In this case, activation by *Snail1* was higher than in SW-480 cells and E-cadherin did not totally block the activity of this promoter (Fig. 4C). Thus, these data corroborate the idea that E-cadherin expression inhibits NF- $\kappa$ B transcriptional activity.

The subcellular localisation of NF- $\kappa$ B was also examined. Cell extracts from cytoplasmic plus membrane and nuclear fractions were prepared. As shown in Fig. 5A, cytoplasmic and membrane proteins (pyruvate kinase and E-cadherin) were effectively separated from nuclear markers [Lamin B and TATA binding protein (TBP)]. Although most of the p65 subunit of NF- $\kappa$ B was detected in the cytosol, a significant fraction was present in the nucleus from SW-480 or SW-480 *Snail1*-transfected cells. This fraction was estimated to be between 10 and 20% of the total p65 by quantification of the different experiments performed (not shown). Overexpression of E-cadherin prevented the detection of NF- $\kappa$ B in this fraction. Similar results were obtained when the presence of  $\beta$ -catenin localisation was determined in these two



**Fig. 3.** E-cadherin controls the transcriptional activity of  $\beta$ -catenin on the fibronectin promoter. (A) *Snail1* and E-cadherin modulate  $\beta$ -catenin and TCF4 transcriptional activity. The activity of a  $\beta$ -catenin–TCF4-dependent promoter (TOP) was determined in SW-480 cells stably transfected with *Snail1*-HA, E-cadherin or both. The results are the average  $\pm$  s.d. of three experiments. (B) Binding of  $\beta$ -catenin to the fibronectin promoter is sensitive to E-cadherin. ChIP assays were carried out as described in the Materials and Methods, immunoprecipitating crosslinked nuclear extracts from SW-480 cells stably transfected with the indicated genes. The average  $\pm$  s.d. of two experiments is shown.





**Fig. 4.** E-cadherin inhibits NF- $\kappa$ B transcriptional activity on the fibronectin promoter. (A) Binding of NF- $\kappa$ B to the fibronectin promoter is sensitive to E-cadherin. ChIP assays were carried out as described in the Materials and Methods, immunoprecipitating crosslinked nuclear extracts from SW-480 cells stably transfected with the indicated genes. Semi-quantitative analysis from one experiment of the three performed (right) or the average  $\pm$  s.d. of quantitative analysis of three experiments (left) is shown. (B) E-cadherin controls p65 association to DNA. Gel shift assays were performed as described in the Materials and Methods, with an oligonucleotide containing the NF- $\kappa$ B-binding element present in the human fibronectin promoter. Nuclear extracts from SW-480 cells transfected with *Snail1* alone or both *Snail1* and E-cadherin were used. In the experiment shown in the left panel, binding of the radioactive probe was competed with a 50- or 100-fold excess of non-radioactive probe containing a consensus binding element for NF- $\kappa$ B, either wild-type (WT) or mutated (MUT). When indicated (right panel), binding was carried out in the presence of an irrelevant IgG or a mAb specific for p65, as indicated in the Materials and Methods. The arrows show the specific band detected with this assay; the arrowhead shows the migration of the free probe. Note that the upper band was not competed either by the NF- $\kappa$ B consensus oligonucleotide or by the p65 antibody; therefore, we considered that it did not correspond to a p65 complex. The results of a representative experiment of the four (right) or five (left) that were performed are shown. (C) *Snail1* and E-cadherin modulate NF- $\kappa$ B transcriptional activity. The activity of an NF- $\kappa$ B-dependent promoter (*NF3*) was determined in SW-480 cells stably transfected with *Snail1*-HA, E-cadherin or both. The same experiment was performed in MiaPaca-2 cells transiently transfected with these two cDNAs. The results correspond to the average  $\pm$  s.d. of three experiments.

fractions: E-cadherin downregulated the amount of  $\beta$ -catenin present in the nuclear fraction.

Immunofluorescence analysis also demonstrated that E-cadherin expression relocated NF- $\kappa$ B and  $\beta$ -catenin to the cytosol. As shown in Fig. 5B, in SW-480 *Snail1*-transfected cells, which grow without establishing cell contacts,  $\beta$ -catenin had a diffuse distribution, labelling the nucleus and the cytosol. Expression of E-cadherin caused the cells to grow in compact colonies even at low cell density and eliminated the nuclear immunoreactivity of  $\beta$ -catenin. Similar results were obtained when p65 was analysed, although in this case only a small signal was detected in the nucleus in SW-480 *Snail1*-transfected cells, confirming our data from the analysis of cell fractions (Fig. 5A).

As shown in Fig. 5B, upon expression of E-cadherin,  $\beta$ -catenin was mainly detected in the cell periphery, in accordance with the previous results of other authors (Stockinger et al., 2001). Surprisingly, p65 was similarly distributed. These analyses were repeated, determining the co-distribution of p65 and E-cadherin (Fig. 5C). Although p65 was mainly detected in the cytosol, it also had a significant colocalisation with E-cadherin in the membrane. Therefore, these results suggest that NF- $\kappa$ B interacts with components of the junctional complex.

#### NF- $\kappa$ B associates with E-cadherin and other junctional components

It has been reported that the p65 subunit of the NF- $\kappa$ B heterodimer associates with  $\beta$ -catenin (Deng et al., 2002). We checked in our cell systems whether p65 interacted with E-cadherin or other components of the adhesion complex. As shown in Fig. 6A by co-immunoprecipitation experiments carried out in SW-480 cells

transfected with E-cadherin and *Snail1*, p65 was associated with E-cadherin and other components of the adhesion complex, such as  $\beta$ -catenin,  $\alpha$ -catenin and p120-catenin. Similar results were obtained in SW-480 E-cadherin-transfected cells (not shown). Co-immunoprecipitation of p65 with  $\beta$ -catenin correlated with the expression of E-cadherin, because  $\beta$ -catenin was not detected in p65 immunoprecipitates from SW-480 *Snail1*-transfected cells, which had very low levels of E-cadherin (Fig. 6A).

Association of p65 with junctional components was also determined by pull-down assays that used as bait GST-fusion proteins containing the cytosolic domain of E-cadherin (cytoE-cadh),  $\beta$ -catenin or p120-catenin (Fig. 6B). The interaction was higher with GST-cytoE-cadh than with the other two fusion proteins. Moreover, association of p65 and GST- $\beta$ -catenin was stimulated by addition of cytoE-cadh (Fig. 6B), suggesting that this protein, or an E-cadherin-bound protein, mediates the interaction of p65 with the junctional complex.

Pull-down assays were also performed with a recombinant p65 protein lacking the last 80 amino acids. This protein did not directly bind either to GST-cytoE-cadh (Fig. 6C), GST- $\beta$ -catenin or GST-p120-catenin (not shown). However, when the binding assays were supplemented with a cell extract, recombinant p65 also associated with GST-cytoE-cadh (Fig. 6C), indicating that interaction between p65 and E-cadherin requires an additional cellular factor.

Co-immunoprecipitation of p65 with endogenous  $\beta$ -catenin and E-cadherin was also detected in IEC-18 cells (Fig. 6D and Fig. 7A,B). Immunofluorescence analysis of the p65 subunit showed the presence of this protein in the cytosol; nuclei were free of immunoreactivity (supplementary material Fig. S5). This diffuse

distribution of p65 in the cytosol made it difficult to detect extensive colocalisation with E-cadherin. However, when the analysis was performed after treating the cells with TNF $\alpha$ , in order to translocate

the cytosolic pool of p65, and washing out with NP-40 prior to fixation, p65 immunoreactivity was seen in the membrane (supplementary material Fig. S5). In any case, our results suggest that only a small part of p65 has this membrane location.

We used IEC-18 cells to verify that only the E-cadherin-associated  $\beta$ -catenin was interacting with p65. For this purpose, we depleted E-cadherin from IEC-18 cell lysates by two successive rounds of immunoprecipitation. In accordance with previous results, p65 was immunoprecipitated with monoclonal antibodies (mAbs) against  $\beta$ -catenin and E-cadherin and not by control IgGs (Fig. 6D). Whereas immunodepletion of E-cadherin greatly decreased the levels of  $\beta$ -catenin (Fig. 6D), it only slightly affected p65 levels, which again suggests that most p65 is not associated with E-cadherin. We found no association between  $\beta$ -catenin not bound to E-cadherin ( $\beta$ -catenin resistant to immunodepletion) and p65 (Fig. 6D), which corroborates the idea that only the membrane-associated pool of  $\beta$ -catenin interacts with p65.

Association of p65 with  $\beta$ -catenin or p120-catenin was also seen in NIH-3T3 cells transfected with E-cadherin, but not in control cells (Fig. 6E). To determine whether this pool of E-cadherin-associated p65 was the same as that bound to the NF- $\kappa$ B inhibitor I $\kappa$ B $\alpha$  (Thanos and Maniatis, 1995), a stabilised form of this protein (S32,36A mutant) was also overexpressed in fibroblasts. As shown in Fig. 6E, ectopic expression of I $\kappa$ B $\alpha$  prevented the co-immunoprecipitation of p65 with components of the adhesion complex, suggesting that both associations are exclusive.

#### Disruption of adherens junctions affects the association of NF- $\kappa$ B with E-cadherin and $\beta$ -catenin

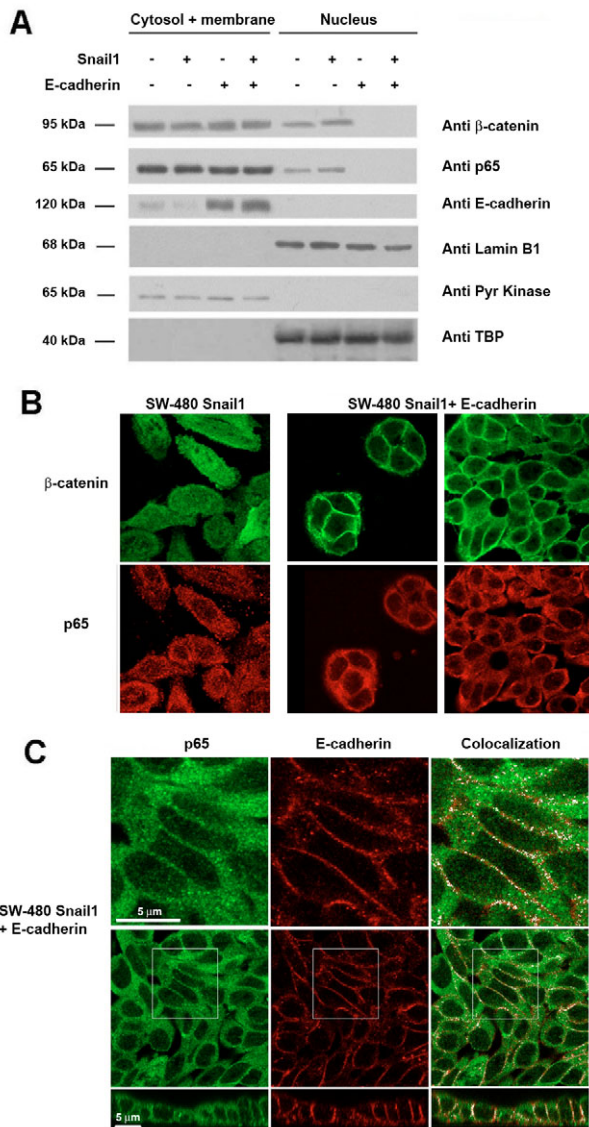
We also examined whether interaction of p65 with adherens-junction components was affected by the disruption of the E-cadherin junctional complex. Transfection of the *K-ras* (*Kras*) oncogene to IEC-18 cells has been reported to promote tyrosine phosphorylation of  $\beta$ - and p120-catenin, and disruption of the intercellular adherens junctional complex (Piedra et al., 2003). We detected the interaction of endogenous p65 and E-cadherin or  $\beta$ -catenin in intestinal epithelial IEC cells, as assayed by co-immunoprecipitation. The levels of E-cadherin or  $\beta$ -catenin present in p65 immunoprecipitates were lower in *K-ras* transfectants than in control IEC-18 cells (Fig. 7A), suggesting that disruption of the junctional complex releases p65 from its interaction with E-cadherin.

To further verify the involvement of E-cadherin in the control of NF- $\kappa$ B activity and p65- $\beta$ -catenin interaction, IEC-18 cells were transfected with an siRNA specific to E-cadherin or an irrelevant siRNA as control. Besides downregulated E-cadherin levels, this E-cadherin siRNA increased the cellular content of fibronectin and *LEF1* RNA in IEC-18 cells (see Fig. 1). As shown in Fig. 7B, transfection of E-cadherin siRNA diminished the interaction between p65 and  $\beta$ -catenin or p120-catenin. Interestingly, the interaction of p65 with I $\kappa$ B $\alpha$  was not modified by E-cadherin siRNA, or by expression of K-ras.

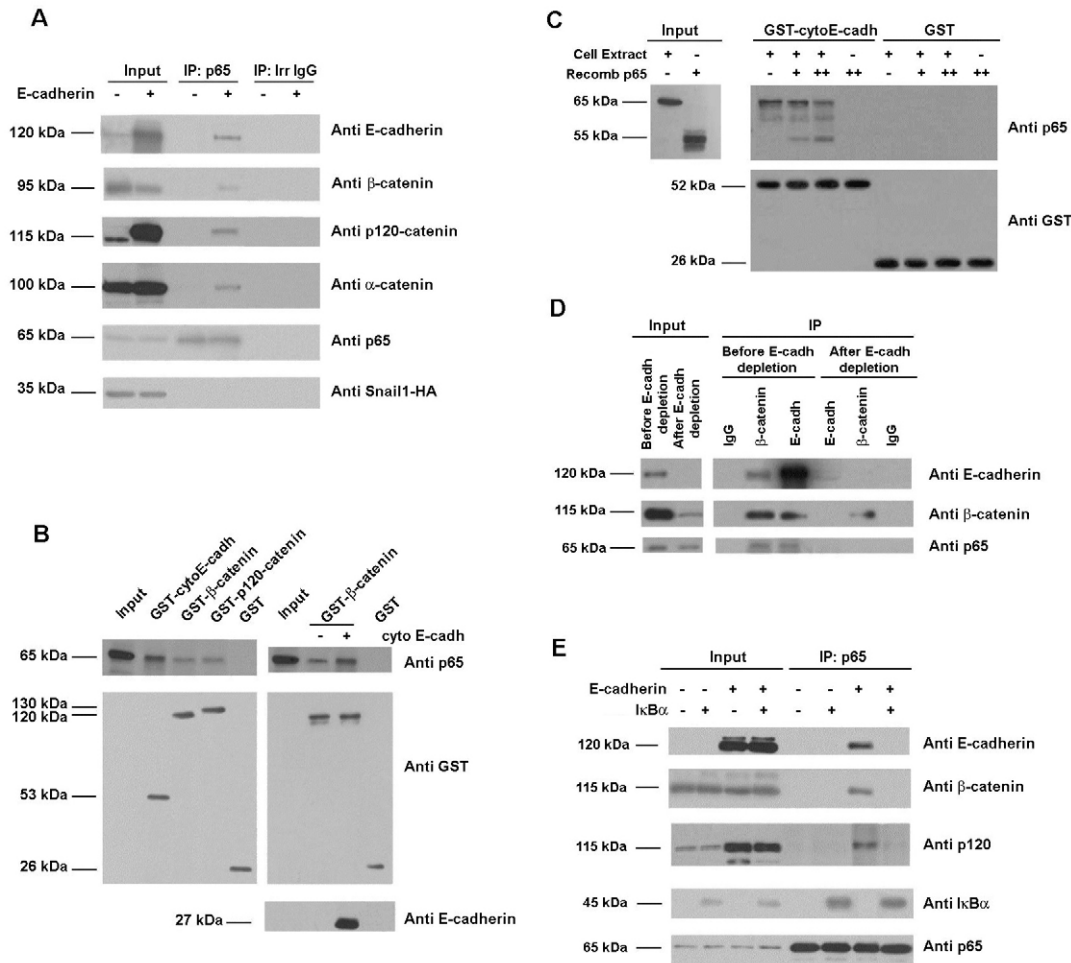
Concomitantly, E-cadherin siRNA upregulated the nuclear levels of p65 (Fig. 7C), determined by cell fractionation and western blot, as well as the activity of the NF- $\kappa$ B-dependent promoter (Fig. 7D). Transfection of E-cadherin siRNA enhanced the response to TNF- $\alpha$  (Fig. 7E), further corroborating that E-cadherin works as a negative effector of this pathway.

#### Discussion

In recent years, the inverse role of Snail1 and E-cadherin in the control of epithelial plasticity has been supported by new evidence (Barrallo-



**Fig. 5.** E-cadherin prevents  $\beta$ -catenin and NF- $\kappa$ B nuclear localisation. (A) Cell fractionation of p65 and  $\beta$ -catenin. Subcellular fractions were prepared from SW-480 cells and analysed by western blot. Lamin B1 and TBP expression were used as nuclear markers; pyruvate kinase and E-cadherin were used as markers for the cytosolic-plus-membrane fraction. (B) Immunolocalisation of p65 and  $\beta$ -catenin is affected by E-cadherin expression. Analysis of  $\beta$ -catenin (green) and NF- $\kappa$ B (red) subcellular localisation was carried out in SW-480 *Snail1*-transfected and SW-480 *Snail1*- and E-cadherin-transfected cells. E-cadherin-positive cells (right) were grown at equal cell density to E-cadherin-negative cells (left), or to a lower density (middle) in order to better visualize cell colonies. The analysis was performed by immunofluorescence using mAbs against  $\beta$ -catenin and NF- $\kappa$ B. No signal was obtained when the same analysis was performed in the absence of primary antibody. (C) Subcellular co-distribution of p65 and E-cadherin. The subcellular distribution of NF- $\kappa$ B p65 subunit (green) and E-cadherin (red) was determined by immunofluorescence in SW-480 E-cadherin-transfected cells as mentioned above, using specific mAbs against these two proteins. The upper row shows an amplified area selected from the panels shown below (boxed). An *xz* section is shown in the bottom row.

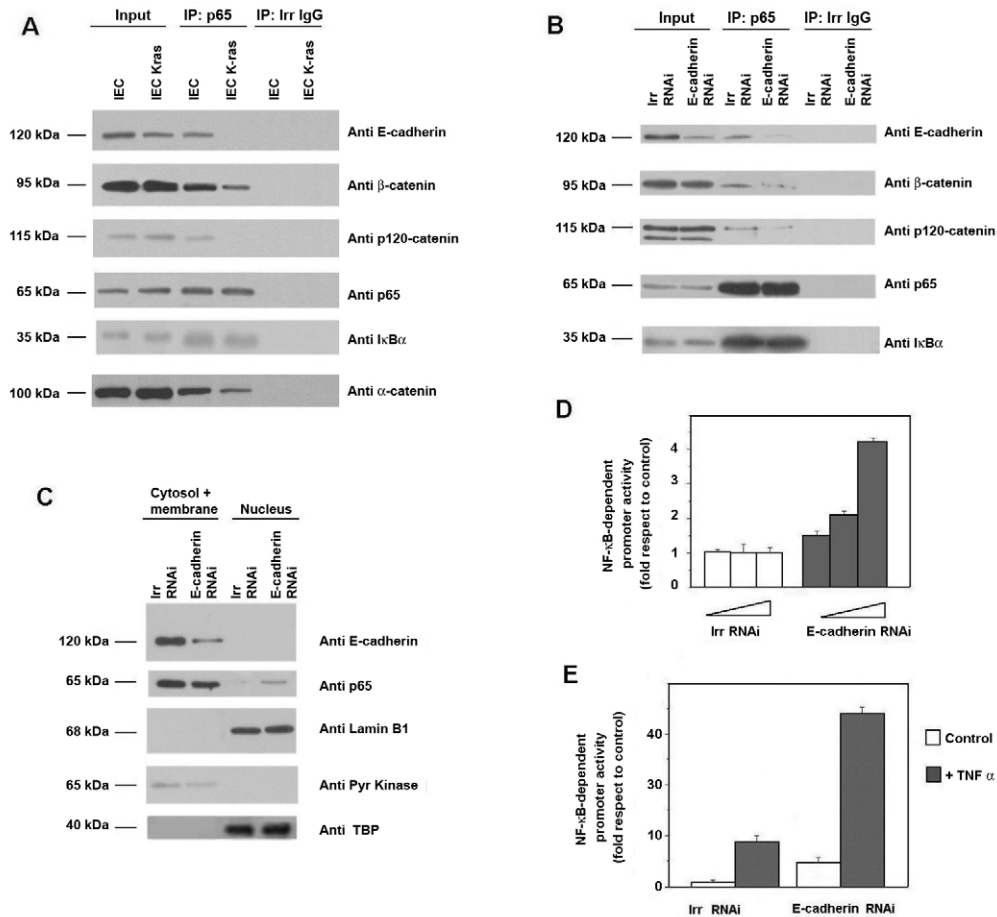


**Fig. 6.** NF- $\kappa$ B associates with E-cadherin and other components of the junctional complex. (A) NF- $\kappa$ B co-immunoprecipitates with E-cadherin and  $\beta$ -catenin. The p65 subunit of NF- $\kappa$ B was immunoprecipitated from whole-cell extracts of SW-480 cells stably transfected with *Snail1*-HA and E-cadherin. The associated proteins were analysed with specific mAbs against E-cadherin, and  $\beta$ -,  $\alpha$ - and p120-catenin. (B) NF- $\kappa$ B associates with E-cadherin and E-cadherin-associated proteins. Pull-down assays were performed by incubating 5 pmol of the different GST-fused proteins with 500  $\mu$ g of cell extracts from confluent SW-480 cells prepared in RIPA buffer. Protein complexes were affinity-purified with glutathione-Sepharose and analysed by western blotting with anti-p65 mAb. Blots were re-analysed with anti-GST antibodies to ensure equal loading of samples. 3% of the total-cell extracts used for the assay was loaded in the input lane. When indicated, 25 pmols of cytoE-cadherin recombinant protein were added to the binding assays. (C) Recombinant purified p65 [1 (+) or 2 (++) pmol] was incubated with 5 pmol of either cytosolic fragment of E-cadherin fused to GST (GST-cytoE-cadh) or GST as a control in the absence or presence of 500  $\mu$ g of SW-480 cell extracts. Protein complexes were affinity-purified with glutathione-Sepharose and analysed by western blotting with anti-p65 mAb. Known amounts of extract (3  $\mu$ g) and recombinant p65 (0.05 pmol, 3 ng) were included in the same blot as controls (Input). (D) NF- $\kappa$ B interacts only with E-cadherin-associated  $\beta$ -catenin. Extracts, prepared as in A, from IEC-18 cells expressing E-cadherin were immunoprecipitated with a mAb specific to E-cadherin in two successive rounds. The presence of p65, E-cadherin and  $\beta$ -catenin was analysed before and after E-cadherin immunodepletion by western blot (input). Extracts before or after immunodepletion were immunoprecipitated with mAbs against E-cadherin,  $\beta$ -catenin or an irrelevant IgG as control and presence of E-cadherin,  $\beta$ -catenin or p65 in the immunocomplex was determined by western blot. (E) I $\kappa$ B $\alpha$  prevents association of NF- $\kappa$ B with E-cadherin. NIH3T3 fibroblasts were transfected with 7.5  $\mu$ g of pcDNA3 control, pcDNA3-E-cadherin or pcDNA3-I $\kappa$ B $\alpha$  S32,S36 mutant (a kind gift from A. Bigas, IDIBELL, Barcelona, Spain). After 48 hours, cell extracts were prepared and the p65 subunit of NF- $\kappa$ B was immunoprecipitated. The presence of E-cadherin, catenins and I $\kappa$ B $\alpha$  in the complexes was analysed by western blot with specific mAbs. The results shown correspond to a representative experiment out of the three performed.

Gimeno and Nieto, 2005). We previously reported that Snail1-induced EMT is associated with the repressive activity of this transcriptional factor, because non-functional *Snail1* mutants, unable to repress E-cadherin gene expression, failed to promote EMT and to activate the transcription of mesenchymal-related genes such as fibronectin and *LEF1* (Dominguez et al., 2003). In this study, we analyse the molecular mechanisms involved in the control of mesenchymal gene expression by E-cadherin. Our results indicate that fibronectin and *LEF1* gene expressions are dependent on the transcriptional activity of  $\beta$ -catenin and NF- $\kappa$ B; these activities are both controlled by the presence of E-cadherin-dependent cell contacts in epithelial cells.

We detected binding of  $\beta$ -catenin and NF- $\kappa$ B to the fibronectin promoter, which correlates with the expression of the fibronectin gene and the activity of its promoter. Previous reports indicate that fibronectin is sensitive to the transcriptional activity of both proteins (Grädl et al., 1999; Chen et al., 2003). However, our results show that factors of the TCF family do not mediate the interaction of  $\beta$ -catenin with the fibronectin promoter. Therefore, an interesting line of research would consist of identifying the factor that mediates the interaction of  $\beta$ -catenin with the fibronectin promoter, as well as characterising other molecular requirements controlling the binding of  $\beta$ -catenin and NF- $\kappa$ B to this promoter.





**Fig. 7.** Disruption of E-cadherin-mediated contacts activates NF- $\kappa$ B transcriptional activity. (A) K-ras expression downregulates E-cadherin interaction with p65. The p65 subunit of NF- $\kappa$ B was immunoprecipitated from total-cell extracts prepared from IEC or IEC *K-ras*-transfected cells. Immunocomplexes were analysed by western blot with mAbs against E-cadherin, and  $\beta$ -,  $\alpha$ - and p120-catenin. The results given correspond to a representative experiment out of the three performed. (B) E-cadherin siRNA affects the association of p65 to junctional-complex components. IEC cells were transfected with an siRNA specific to E-cadherin or with an irrelevant siRNA as control. Total extracts were prepared, the p65 subunit of NF- $\kappa$ B was immunoprecipitated and immunocomplexes were analysed by western blot with mAbs against E-cadherin, and  $\beta$ -,  $\alpha$ - and p120-catenin. The results given correspond to a representative experiment out of the three performed. (C) E-cadherin siRNA increases p65 nuclear levels. Presence of p65 in the nucleus was determined by western blot analysis of cell fractions prepared from cells transfected with E-cadherin siRNAs or irrelevant siRNAs (Irr), as indicated above. Lamin B1 and TBP or pyruvate kinase were used as nuclear and cytosolic markers, respectively. (D,E) E-cadherin siRNA increases NF- $\kappa$ B transcriptional activity. IEC cells were transfected with irrelevant or E-cadherin siRNAs and NF3 reporter plasmid. Luciferase activity was determined after 48 hours and is shown as the average  $\pm$  s.d. of three experiments performed in triplicate. In E, cells were deprived of serum for 16 hours and incubated with TNF $\alpha$  (20 ng/ml) for 6 hours before analysis of *NF3* promoter activity.

Our results indicate that E-cadherin expression negatively controls the transcriptional activity of both  $\beta$ -catenin and NF- $\kappa$ B, and the transcription of mesenchymal genes. Curiously, NF- $\kappa$ B and  $\beta$ -catenin might also indirectly affect E-cadherin expression, because they are involved in the activation of Slug (Snai2), ZEB1 and ZEB2, three E-cadherin repressors also activated during EMT (Conacci-Sorrell et al., 2003; Chua et al., 2007). This hypothesis, suggesting a mutual opposition between these transcriptional factors and E-cadherin, is supported by data from other authors (Conacci-Sorrell et al., 2003) and explains the high degree of plasticity of the epithelial phenotype.

The interference by E-cadherin of  $\beta$ -catenin-dependent gene expression has been extensively studied by many authors. It is well accepted that the  $\beta$ -catenin–E-cadherin complex constitutes the structural core of the adherens contacts, and impedes  $\beta$ -catenin transfer to the nucleus and its transcriptional activity (Orsulic et al., 1999). However, additional factors are also involved in the

control of gene transcription by E-cadherin, because the effects of the loss of this protein on tumour progression are not entirely  $\beta$ -catenin dependent (Herzig et al., 2007). In this respect, an inverse correlation between E-cadherin levels and NF- $\kappa$ B activity has been reported in several systems (Huber et al., 2004; Kuphal et al., 2004; Kuphal and Bosserhoff, 2006; Shin et al., 2006), although the mechanism underlying this effect has not yet been clarified. The results given in this article are the first evidence that NF- $\kappa$ B, similar to  $\beta$ -catenin, is regulated by E-cadherin-dependent immobilisation at the membrane. Although this interaction explains the negative effect of E-cadherin on NF- $\kappa$ B-dependent transcription, we cannot rule out the possibility that E-cadherin also affects other factors required for the activity of this transcription factor.

Competition between the NF- $\kappa$ B and  $\beta$ -catenin transcriptional pathways was reported by Deng et al. (Deng et al., 2002), whose conclusion was based on the indirect interaction detected between



the two proteins. Our results showing that  $\beta$ -catenin and p65 are both bound to the fibronectin promoter during gene activation are compatible with the hypothesis that the simultaneous activation of both pathways is required for the activation of specific genes during EMT (Brabletz, 2005; Huber et al., 2005). According to our data, NF- $\kappa$ B transcriptional activity is mainly inhibited by the adherens-junction-associated pool of  $\beta$ -catenin and not by the transcriptional nuclear pool. Because the interaction between NF- $\kappa$ B and  $\beta$ -catenin does not seem to be direct, it is very likely that the factor mediating this binding is not present in the nucleus and only acts when  $\beta$ -catenin is associated to the junctional complex, where this putative factor might be located. Moreover, this indirect interaction would help to explain why the association of NF- $\kappa$ B with the junctional-complex components is affected in different ways by E-cadherin depletion and K-ras-triggered phosphorylation of catenins.

Our results suggest that the pool of NF- $\kappa$ B bound to E-cadherin is much smaller than the pool bound to  $\kappa$ B $\alpha$ . However, this membrane pool has a functional relevance, because disruption of the junctional complex, for instance caused by depletion of E-cadherin, upregulates NF- $\kappa$ B transcriptional activity. This effect is specific, because this siRNA does not alter the association of p65 with  $\kappa$ B $\alpha$ . Our results suggest that this membrane-associated pool is the one that is mobilised during EMT and is relevant for the expression of mesenchymal genes.

Given the results in this study, we propose a model to explain how E-cadherin inhibits mesenchymal gene expression and, in consequence, EMT. In epithelial cells with mature adherens junctions, p65 and  $\beta$ -catenin are stabilised in a membrane structure that includes E-cadherin. Disruption of contacts by E-cadherin repression, as occurs during EMT, releases these signalling molecules to the cytosol and therefore enables their translocation to the nucleus. According to this model, de-assembly of E-cadherin-mediated contacts would be necessary, but not sufficient, for full mesenchymal gene expression because, before reaching the nucleus,  $\beta$ -catenin and NF- $\kappa$ B are modulated by additional mechanisms. These mechanisms might also be affected by EMT inducers, such as Snail1. Once in the nucleus, p65 and  $\beta$ -catenin would mediate the activation of specific mesenchymal genes. Gene-target specificity, how both signals cooperate and the possible additional effects of Snail1 are subjects for future studies. However, the synchronic binding of  $\beta$ -catenin and p65 to the fibronectin promoter upon downregulation of E-cadherin might be a requirement for the specific activation of fibronectin, and possibly other mesenchymal genes, during EMT.

## Materials and Methods

### Cultured cells

LS174T is a colon-cancer cell line that, although its E-cadherin mRNA levels are comparable with other epithelial cell lines, is deficient for E-cadherin protein expression. This cell line has high transcriptional activity of the  $\beta$ -catenin-TCF4 complex (van de Wetering et al., 2003). LS174T-inducible clones obtained from H. Clevers (Hubrecht Laboratories, Utrecht, The Netherlands) were grown in selective medium as reported previously (van de Wetering et al., 2003) and transfected with pcDNA3-Snail1-IRES-neo or pcDNA3-Snail1-IRES-hrGFP by means of a Lipofectamine Plus kit from GIBCO. Transfected cells were selected in medium containing 300  $\mu$ g/ml of G-418 (geneticin; GIBCO) or using a fluorescence-activated cell sorter (FACS), and individual clones were isolated and grown under standard conditions. Clones expressing Snail1 were named S and control plasmid C. Other cell lines used in these studies were MiaPaca-2, a pancreas cell line deficient in E-cadherin (Batlle et al., 2000); intestinal IEC-18 cells either transfected with a control plasmid or with *K-ras* oncogene (Piedra et al., 2003); and SW-480-ADH (Pálmer et al., 2001), a cell population expressing low levels of E-cadherin prior to confluence. To obtain SW-480 double transfectants, SW-480-ADH cells were transfected with E-cadherin cDNA in the eukaryotic vector pBATEM2 (Nose et al., 1988) (kindly

provided by M. Takeichi, Kyoto University, Kyoto, Japan), using Lipofectamine Plus (Invitrogen). Stable transfectants were obtained after selection with 2 mg/ml G-418 and screened by western blot and immunofluorescence. The clones with higher E-cadherin expression were selected for subsequent experiments. Next, cells were retrovirally transduced with murine *Snail1* cDNA tagged at the 3' end with the sequence encoding the influenza haemagglutinin 12 amino acid peptide cloned into the pRV-IRES-GFP retroviral vector (E-cadherin-Snail1-HA cells) or with the empty pRV-IRES-GFP vector (E-cadherin cells). Retroviral infection was performed as described elsewhere (Peiró et al., 2006). Transduced (GFP+) cells were sorted by an Epics Altra HSS (Beckman-Coulter) and the pool of infected cells was used for further studies. The generation and characteristics of cell clones of the intestinal epithelial cell line HT29-M6, showing doxycycline-regulated expression of Snail1-HA, were reported by Batlle et al. (Batlle et al., 2000).

### DNA constructs and promoter assays

An expression plasmid containing an siRNA for human E-cadherin was prepared as indicated. Briefly, two complementary oligonucleotides were annealed and cloned in the pSUPER basic vector at *Bgl*II-*Xho*I sites. Forward oligonucleotide was 5'-GATCCCCCGATCAGAATGACAACAATTCAAGAGATTGTTGTCTATCTGATCGGTTTTTC-3', where tandem sense-antisense 19 b sequences from the human E-cadherin cDNA are underlined. This sequence was conserved in rat E-cadherin with the exception of two bases, placed at positions 2 and 18. A scrambled sequence was used as control. The construction was verified by sequencing.

Generation of pGL3 luciferase reporter vector (Promega) containing human *LEF1* promoter (-735/+1077) has been described (Dominguez et al., 2003). A -341/+265 fragment of human fibronectin promoter was obtained by PCR using sense 5'-CCCCACGCGTACACAAGTCCAGCCACTCCC-3' and antisense 5'-GTTGAG-ACGGTGGGGAGAG-3' oligonucleotides. NF3 plasmid, an NF $\kappa$ B-sensitive plasmid containing three binding sequences for this transcriptional factor upstream from a luciferase reporter gene, was kindly provided by M. Fresno (Universidad Autónoma de Madrid, Spain). Reporter assays were performed as described elsewhere (Batlle et al., 2000; Barberà et al., 2004). When indicated, cells were deprived of serum for 16 hours and supplemented with TNF $\alpha$  (kindly provided by C. de Bolós, IMIM), then resuspended at 10  $\mu$ g/ml in PBS + 0.1% BSA to a final concentration of 20 ng/ml for 6 hours. The use of plasmids encoding VP16-Rel and VP16-TCF4 fusion proteins and  $\Delta$ TCF4 mutant has been reported elsewhere (Tan et al., 2001; Barberà et al., 2004).

### RNA analysis

mRNA was obtained following a standard protocol (Guaita et al., 2002) or using GenElute Mammalian Total RNA Miniprep kit (Sigma-Aldrich). Normally, 50 ng of purified mRNA were analysed with Super-Script One-Step RT-PCR with Platinum Taq Polymerase (GIBCO). RT-PCR products were separated on 1.5% agarose/Tris-Acetate-EDTA gels. For E-cadherin, fibronectin, LEF1, murine Snail1 and cyclophilin analysis, the primers used are indicated in supplementary material Table S1 or in Guaita et al. (Guaita et al., 2002).

For quantitative mRNA detection, 250 ng of total RNA extracted with the RNA kit (Sigma) were analysed using QuantiTect SYBR Green RT-PCR (Qiagen) in triplicate. RT-PCR and data collection were performed on ABI PRISM 7900HT. All quantifications were normalised with either endogenous cyclophilin or HPRT. The relative value of each target gene compared with the calibrator for that target is expressed as  $2^{-(Ct-Cc)}$ , where *Ct* and *Cc* are the mean threshold cycle differences after normalising to control. For primer sequences, see supplementary material Table S1.

### ChIP assays

ChIP assays were performed in SW-480, SW-480-Snail1, SW-480-Ecadherin or SW-480-Snail1-Ecadherin cells, essentially as described by Peiró et al. (Peiró et al., 2006).  $15 \times 10^6$  cells were cross-linked with 1% formaldehyde. They were lysated in SL buffer (50 mM Tris pH 8, 2 mM EDTA, 0.1% NP40, 10% glycerol), incubated for 10 minutes on ice and centrifuged for 5 minutes at 2300 g. Supernatant was discarded and pellet resuspended in SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris pH 8) for 10 minutes at room temperature. Cell lysates were sonicated to generate fragments of DNA from 200 to 1500 bp. Protein concentration was determined by Bradford and equal amounts of protein in 100  $\mu$ l of SDS lysis buffer extract were diluted 1/10 with IP buffer (0.001% SDS, 1.1% Triton X-100, 16.7 mM Tris pH 8, 2 mM EDTA, 1.2 mM EGTA, 167 mM NaCl). Immunoprecipitation was carried out with  $\beta$ -catenin mAb (BD Biosciences), p65-NF- $\kappa$ B polyclonal antibody (Santa Cruz Biotechnology) or mouse IgG (Dako) as a control. Samples were treated with elution buffer (100 mM Na<sub>2</sub>CO<sub>3</sub>, 1% SDS) and incubated at 65°C overnight to reverse formaldehyde cross-linking. Samples were digested with proteinase K and RNase, and DNA was purified by the GFX PCR DNA and Gel Band Purification kit (Amersham Biosciences). Fibronectin promoter regions (GeneBank AC012462) were detected by PCR using two specific primers that amplify the proximal region (5'-GTTGAGACGGTGGGGGAGA-3', corresponding to sequence 70304-286; and 5'-CCGTCCCTTCCCCA-3', corresponding to 70423-37) or, as control, two primers that amplify a fragment 2-kb upstream of the fibronectin proximal promoter (5'-TCCTTCCCCAGAAATCAATGAA-3', 72512-491; and 5'-GGGAAGCCGAGTG-TTCTCTTCC-3', 72404-24).

### Electrophoretic mobility shift assays

Nuclear extracts were prepared by suspending pelleted cells with two volumes of buffer I (10 mM HEPES pH 7.6, 1.5 mM MgCl<sub>2</sub>, 10 mM KCl, 0.5 mM DTT, 0.5% NP-40 and a cocktail of proteases and phosphatase inhibitors). After incubation for 10 minutes on ice and 10 minutes of centrifugation at 2300 g, the resulting pellet was resuspended in 2 volumes of buffer II (20 mM HEPES pH 7.6, 1.5 mM MgCl<sub>2</sub>, 840 mM KCl, 0.5 mM DTT, 0.2 mM EDTA, 25% glycerol, and a cocktail of protease and phosphatase inhibitors). Subsequently, the samples were kept on ice for 20 minutes and centrifuged for 30 minutes at 2300 g. The supernatants obtained were dialysed for 15 hours at 4°C in buffer III (20 mM HEPES pH 7.6, 100 mM KCl, 0.5 mM DTT, 0.2 mM EDTA, 25% glycerol), and the protein concentration was analysed by Bradford and used as a nuclear extract. Assays were performed essentially as described (Batlle et al., 2000). The <sup>32</sup>P-labelled double-stranded oligonucleotide 5'-ggggaggagaGGAACCCAGGcgagc-3' (corresponding to nucleotides +24/+53 of the human fibronectin promoter, in relation to the transcription start site) was used as a radioactive probe. In this oligonucleotide, the putative NF-κB-binding element is given in capital letters. When mentioned, binding of the radioactive probe was competed with a 50- or 100-fold excess of wild-type (5'-agttgaGGGA-CCTTCCcagge) or mutated (5'-agttgaGGAGATCTGGCcagge-3', where mutated nucleotides are underlined) double-stranded oligonucleotides corresponding to the DNA consensus binding element for NF-κB (Huang et al., 2006). When indicated, binding reaction was supplemented with 10 μg of irrelevant IgG or anti-p65 polyclonal antibody (from Santa Cruz Biotechnology, sc-372) and incubated for 15 minutes prior to the addition of the radiolabelled probe.

### Preparation of cell extracts and immunoprecipitation

Total cell extracts were prepared by homogenising cells in RIPA buffer (25 mM Tris-HCl pH 7.6, 200 mM NaCl, 1 mM EGTA, 0.5% sodium deoxycholate, 0.1% SDS, 1% Nonidet P-40) supplemented with protease inhibitors (0.3 μM Aprotinin, 1 μM Leupeptin, 1 μM Pepstatin, 1 mM Pefabloc) and phosphatase inhibitors (10 mM NaF, 0.1 mM sodium orthovanadate). After passing cells ten times through a 20-gauge syringe, extracts were left on ice for 20 minutes and centrifuged at 20,000 g for 5 minutes at 4°C. To separate nucleus from membrane and cytosol, cells were lysed in RIPA buffer and resuspended carefully with a micropipette. Integrity of the nuclei was verified by staining with DAPI and viewing them in the microscope. After centrifuging at 300 g for 10 minutes, the supernatant was considered to be the cytosol and membrane fraction. As shown below, this fraction was free of nuclear markers. The pellet was resuspended in the same volume of RIPA buffer, passed ten times through a 20-gauge syringe and centrifuged at 20,000 g for 10 minutes. The supernatant was considered to be the nuclear fraction. Equivalent amounts of protein extract were loaded in each condition. Cell fractions were analysed by western blot using a specific polyclonal antibody against fibronectin (Abcam) or mAbs against NF-κB p65 subunit, E-cadherin, β-catenin, TBP (all from BD Biosciences), Lamin B1 (Abcam), α-tubulin, actin or pyruvate kinase (Pyr kinase) (Sigma).

Proteins were immunoprecipitated from cell extracts (300–500 μg), using 4 μg/ml of antibody to p65 (sc-109, Santa Cruz) or E-cadherin (from BD Biosciences), for 16 hours at 4°C and then collected using 30 μl of γ-bind G-Sepharose (GE Healthcare). Immunoprecipitates were washed three times with lysis buffer and eluted with electrophoresis sample buffer. Immunoprecipitated proteins were analysed by western blot by using specific antibodies against p65, E-cadherin, β-catenin, α-catenin, p120-catenin, IκBα (Santa Cruz Biotechnology) or HA (Roche 3F10). 3% of cell extracts used for IP were loaded into the input lane as control. Serial immunoblots were performed after stripping the membranes.

### Protein-binding assays

Pull-down assays were performed using 5 pmols of purified recombinant proteins fused to GST and 500 μg of cell extracts from SW-480 and NIH3T3, as reported previously (Solanas et al., 2004). When indicated, a recombinant p65 protein lacking the last 80 amino acids (Active Motif, Carlsbad, CA) was added to the assay. Glutathione-Sepharose-bound proteins were analysed by western blotting with specific mAbs against the NF-κB p65 subunit or E-cadherin. The polyclonal antibody to the GST protein was from GE Healthcare.

### Immunofluorescence

SW-480 cells, plated on coverslips, were washed with PBS, fixed with 4% PFA for 15 minutes, washed twice with PBS, permeabilised with 0.2% Triton for 10 minutes and washed exhaustively with PBS (five times) to ensure rinsing. Cells were blocked with 5% non-fat milk diluted in TBS for 1 hour. A dilution of the primary antibodies in 3% BSA-TBS (1/400 for anti-p65 from Santa Cruz Biotechnology and 1/40 for anti-E-cadherin from BD Biosciences) was used to incubate the coverslips overnight at 4°C. After being washed with PBS, cells were incubated with secondary antibodies (diluted 1/500 in PBS) for 1 hour at room temperature. Cells were washed again and coverslips were mounted on glass slides with Mowiol.

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