

**PRO-TUMORIGENIC EFFECTS OF SNAIL-EXPRESSION FIBROBLASTS ON  
COLON CANCER CELLS**

**Alberto Herrera<sup>1</sup>, Mercedes Herrera<sup>1</sup>, Lorena Alba-Castellón<sup>2</sup>, Javier Silva<sup>1</sup>,  
Vanesa García<sup>1</sup>, Jordina Loubat-Casanovas<sup>2</sup>, Ana Álvarez-Cienfuegos<sup>3</sup>, José  
Miguel García<sup>1</sup>, Rufo Rodríguez<sup>4</sup>, Beatriz Gil<sup>1</sup>, M<sup>a</sup> Jesús Citores<sup>5</sup>, M<sup>a</sup> Jesús  
Larriba<sup>6</sup>, J. Ignacio Casal<sup>7</sup>, Antonio García de Herreros<sup>2,8</sup>, Félix Bonilla<sup>1\*</sup>,  
Cristina Peña<sup>1\*</sup>**

1 Department of Medical Oncology, Hospital Universitario Puerta de Hierro de  
Majadahonda, Majadahonda, Madrid, Spain.

2 Programa de Recerca en Càncer, Institut Hospital del Mar d'Investigacions Mèdiques,  
Barcelona, Spain.

3 Department of Molecular Immunology, Hospital Universitario Puerta de Hierro de  
Majadahonda, Majadahonda, Madrid, Spain.

4 Department of Pathology, Hospital Virgen de la Salud, Toledo, Spain.

5 Department of Tumour Immunology, Hospital Universitario Puerta de Hierro,  
Majadahonda, Madrid, Spain.

6 Instituto de Investigaciones Biomédicas Alberto Sols, Consejo Superior de  
Investigaciones Científicas - Universidad Autónoma de Madrid, Madrid, Spain.

7 Functional Proteomics Laboratory, Centro de Investigaciones Biológicas (CIB-CSIC),  
Madrid, Spain.

8 Departament de Ciències Experimentals i de la Salut, Universitat Pompeu Fabra,  
Barcelona, Spain.

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\*Corresponding authors: Department of Medical Oncology, Hospital Universitario  
Puerta de Hierro de Majadahonda, Majadahonda, Madrid, Spain. E-mail: CP:  
cpena@idiphim.org, FB: felix.bonilla@salud.madrid.org. Tel: +34-91 1916770.

### **Impact Statements**

Snail1 expression in tumour stroma is associated with a lower specific survival in colon cancer patients. The present study, using established human primary normal and cancer associated fibroblasts, normal and tumour colon cancer samples, ectopic Snail1 modified fibroblasts and colon cell and xenografted nude mice models, reveals, for the first time, that Snail1 is necessary for the pro-tumorigenic effects of fibroblasts by controlling migration and proliferation on colon cancer cells.

## ABSTRACT

Snail1 is a transcriptional factor that plays an important role in epithelial-mesenchymal transition and in the acquisition of invasive properties by epithelial cells. In colon tumours, Snail1 expression in the stroma correlates with lower specific survival of cancer patients. However, the role(s) of Snail1 expression in stroma and its association with patients' survival have not been determined. We used human primary Carcinoma Associated Fibroblasts (CAFs) or Normal Fibroblasts (NFs) and fibroblast cell lines to analyze the effects of Snail1 expression on the pro-tumorigenic capabilities in colon cancer cells. Snail1 expression was higher in CAFs than in NFs and, as well as  $\alpha$ -SMA, a classic marker of activated CAFs. Moreover, in tumour samples from 50 colon cancer patients, *SNAIL1* expression was associated with expression of other CAF markers, such as  $\alpha$ -SMA and FAP. Interestingly, co-culture of CAFs with colon cells induced a significant increase in epithelial cell migration and proliferation, which was associated with endogenous *SNAIL1* expression levels. Ectopic manipulation of Snail1 in fibroblasts demonstrated that Snail1 expression controlled migration as well as proliferation of co-cultured colon cancer cells in a paracrine manner. Furthermore, expression of Snail1 in fibroblasts was required for the co-adjuvant effect of these cells on colon cancer cell growth and invasion when co-xenografted in nude mice. Finally, cytokine profile changes, particularly MCP3 expression, in fibroblasts are put forward as mediators of Snail1-derived effects on colon tumour cell migration. In summary, these studies demonstrate that Snail1 is necessary for the pro-tumorigenic effects of fibroblasts on colon cancer cells.

## INTRODUCTION

Snail1 transcriptional factor is a key factor for triggering Epithelial-Mesenchymal Transition (EMT), a process characterized by the loss of epithelial characteristics and the acquisition of mesenchymal traits and higher motility.<sup>1</sup>

Snail1 expression is observed at different stages during embryo development and is required for numerous developmental processes.<sup>2</sup> Snail1 expression and EMT features have also been observed in breast, prostate, lung, ovarian, melanoma, colon and oesophageal cancers.<sup>3-13</sup> In most cases, Snail1 is detected in the invasive areas of the tumour. An analysis of colon tumours has shown that Snail1 is mainly detected in the tumour stroma, in cells with a fibroblast phenotype. Furthermore, Snail1 expression in tumour stroma is associated with shorter specific survival in colon cancer patients.<sup>12</sup> In addition, we recently noted the paracrine effects of Snail1 expression in human colon cells, showing down-regulation of *CDH1* and *VDR* gene expression when co-cultured with Snail1 over-expressing colon cancer cells.<sup>14</sup> However, the molecular mechanism underlying this association is not yet clear.

Taking all these data together, this study aimed to characterize Snail1 as a novel enhancer of paracrine-derived effects on colon cancer cell migration and proliferation and to describe the novel cytokine profile induced by Snail1 expression in fibroblasts.

## **MATERIAL AND METHODS**

### **Establishment of primary colon NFs and colon cancer CAFs**

For CAF and NF establishment, fresh tumour and normal colon mucosa samples were obtained from Puerta de Hierro University Hospital, Majadahonda, and Virgen de la Salud Hospital, Toledo, between January and December 2011. Informed written consent was obtained from all participants after an explanation of the nature of the study, as approved by the Research Ethics Board of Puerta de Hierro Majadahonda University Hospital. For CAF establishment and growth details, see Supplementary Information.

To evaluate the CAF enrichment of the culture, Vimentin and Pan Cytokeratin (PanCK) were analysed for immunofluorescence (Supplementary Figure 1A).

### **Normal and colon tumour human samples**

The study is based on two consecutive series of 50 and 65 patients who had surgery for colon cancer between January 1998 and January 2003. The first patient cohort was a subgroup of a cohort previously described by Peña et al., 2005;<sup>15</sup> and the second, a subgroup of a cohort previously described by Herrera et al., 2013.<sup>16</sup> Informed written consent was obtained from all participants, as approved by the Research Ethics Board of Puerta de Hierro Majadahonda University Hospital. Colon cancer-derived samples were obtained immediately after surgery, snap-frozen in liquid nitrogen and stored at -80°C until processing.

### **RNA treatment and Quantitative Real-Time PCR (RT-PCR)**

RNA extraction, target gene mRNA quantification and synthesis of cDNA were performed as described elsewhere.<sup>14</sup>

## **Immunohistochemistry and immunofluorescence analysis in human and mouse samples**

The following antibodies were used in this study: SNAI1 mAb EC3 antibody,<sup>17</sup> anti-alpha smooth muscle actin antibody [1A4] (Abcam), polyclonal antibody to fibroblast activation protein (FAP) alpha (Imgenex, San Diego, CA, USA), monoclonal antibodies anti-pan cytokeratin (ab6401, Abcam) and anti-vimentin (ab92547, Abcam); anti Ki67 (ab21700, Abcam) and anti E-cadherin (Transduction Labs 610182). Procedures were performed as described and Supplementary Information.<sup>12, 18</sup>

## **Co-culture of tumour colon cells and human colon primary CAFs or fibroblasts cell lines for migration assays**

Colon tumour cell lines were co-cultured with colon primary CAFs or fibroblasts cell line for migration assays. Cells were co-cultured with physical separation, in a setting in which primary CAFs were seeded in the lower compartment of a transwell system and colon cells in the upper one. After 48 h, epithelial cells that had reached the lower surface of the filter were measured by fluorescence. For procedure and growth details, see Supplementary Information.

## **Co-culture of tumour colon cells and fibroblast cell lines for proliferation assays**

Proliferation assays were carried out in 24-well plates by co-culturing Snail1-expressing cells in the upper chamber of a 0.4 µm trans-well culture (Becton Dickinson) and SW480-ADH-GFP cells in the lower one. Cells were grown in DMEM containing 5% FBS for 6 days; proliferation rate was analyzed at 3 and 6 days by a fluorescence reader (Tecan Infinite 200 Pro).

### **FACS analysis of the cell cycle of colon cancer cells**

Tumour colon cells were incubated with conditioned medium from fibroblasts or CAFs for 24h. Afterwards, cells were fixed in 70% ethanol and treated with 0.2 mg/ml RNase (Qiagen) and propidium iodide (Sigma Chemical Co.) at 20 µg/ml in PBS. Cell DNA content was evaluated in a MACSQuant Analyzer (Miltenyi Biotech). See details in Supplementary Information.

### **In-vivo xenograft tumour model**

One million HT-29 M6 cells plus  $10^5$  MEFs were subcutaneously injected into the flank of 8-week-old athymic female nude mice. Growth of the tumour was followed every other day after the first week. The study was approved by the Animal Research Ethical Committee of the PRBB. N=6.

### **Human Protein Cytokine Array**

Conditioned medium was harvested from fibroblasts and incubated with membranes containing an array of 80 human cytokine antibodies (RayBio® Human Cytokine Antibody Array 5, RayBiotech Inc.). The bound complex was detected with Odyssey Infrared Imaging System (Li-Cor) at 800 nm, using Streptavidin DyLight 800 Conjugates (Rockland Immunochemicals). Details are described in Supplementary Information.

### **Colon cell migration with recombinant protein**

Colon tumour cell migration assays, developed with medium supplemented with the corresponding cytokines, were conducted as described above and in Supplementary Information.

## RESULTS AND DISCUSSION

Snail1 gene (*SNAIL*) expression was determined in Cancer-Associated Fibroblasts (CAFs), isolated from 13 colon cancer patients, and compared with the expression in fibroblasts isolated from normal colon mucosa of 5 colon cancer patients (NFs). *SNAIL* mRNA expression was detected in CAFs from all patients' samples, with relative expression levels between 2.81 and 0.34. Interestingly, expression levels of mRNA *SNAIL* were higher in CAFs than in fibroblasts from normal colon mucosa (Figure 1A). Thus, these data revealing a significant increase of *SNAIL* expression in CAFs vs NFs suggest that Snail1 can be considered a CAF marker. As a control,  $\alpha$ -SMA, a well-studied marker of these fibroblasts,<sup>19</sup> was also up-regulated in CAF vs NF (Supplementary Figure 1B).

In view of these data, the association between *SNAIL* expression and CAF markers was studied in normal and tumour samples of 50 colon cancer patients. As shown in Figure 1B and 1C, *SNAIL* expression was associated with  *$\alpha$ -SMA* and *FAP* expression markers. Moreover, IHC analysis of *SNAIL*,  $\alpha$ -SMA and FAP in human colon tumour samples showed staining that overlapped the three markers (Supplementary Figure 1C).

To investigate the relevance of Snail1 expression in the properties of CAFs, we performed migration assays in Boyden chambers using colon cancer cells co-cultured with primary CAFs isolated from 11 colon cancer patients. As expected, CAFs enhanced LIM1215 cell migration between 8- and 140-fold more than cells cultured without fibroblast stimulation. Interestingly, statistical analysis showed direct correlation between mRNA *SNAIL* expression levels in primary human CAFs and its ability to induce colon cancer cell migration (Figure 1D). This correlation was also



validated when another colon cancer cell line, SW480-ADH, was used (Supplementary Figure 1D).

These results suggest that the up-regulation of *SNAIL* in tumour stroma enhances colon cancer cell migration. Therefore, to verify this conclusion we used different fibroblast cells in which we ectopically manipulated Snail1 levels (Supplementary Figure 2A). Snail1 over-expression in 1.BR3.G human immortalized fibroblasts up-regulated the capacity of these cells to increase the migration of SW480-ADH and LIM1215 cells over mock-transfected 1.BR3.G cells (Figure 1E and Supplementary Fig 2B, respectively).

The effect of Snail1 depletion in fibroblasts was also analyzed. We took advantage of the availability of murine embryo fibroblasts (MEFs) derived from a mouse bearing a Snail1-floxed form of this gene (Supplementary Figure 2A).<sup>20</sup> After depletion of these genes by expression of Cre recombinase, the effect of wild-type and Snail1 KO MEFs on SW-480 ADH migration was determined. As shown in Figure 1F, SW480-ADH-Mock cell migration was significantly less when co-cultured with Snail1 KO MEFs than when co-cultured with wt MEFs. The different effect on colon cancer cell migration of wt and Snail KO MEFs was also confirmed when using HT29-M6 or LIM1215 cells (Supplementary Figure 2C and 2D).

The increase in migration of target cells when co-cultured with cells in which Snail1 was ectopically expressed was not restricted to fibroblasts: Snail1 expression either in SW480-ADH-Snail1 or in HT-29-Snail1 colon cancer cells induced a significant increase in the migration of co-cultured SW480-ADH Mock cells (Supplementary Figure 2E and 2F).

These experiments demonstrate that Snail1 expression induces paracrine pro-migratory effects on colon cancer cells. We also analyzed whether the proliferation rates of epithelial cells increased when co-cultured with Snail1-expressing cells. For this, colon cells were co-cultured in 0.4µm Boyden chambers with Snail1-expression or mock fibroblasts. Proliferation rate was measured at 3 and 6 days of the co-culture by fluorescence. An increase in the SW480-ADH cell proliferation rate was observed when co-cultured with 1.BR3.G-Snail1 human fibroblasts (Figure 1G). SW480-ADH cell proliferation also increased when Snail1 was expressed in the same cells (Supplementary Figure 2G).

In line with these results, Snail1-expressing fibroblasts statistically increased the number of SW480-ADH Mock cells in the S/M phase (Supplementary Figure 2H). In parallel, CAFs from nine colon cancer patients, with large differences in their mRNA *SNAIL1* expression levels, offered similar results. There was direct correlation between *SNAIL1* levels of CAFs and the percentage of SW480-ADH cells in the S/M phase (Figure 1H).

The in-vivo significance of these findings was also explored in a xenograft mouse model. HT29M6 cells were subcutaneously co-injected with wild-type or Snail KO MEFs in 6 mice. As expected, Snail1 immunostaining showed lower Snail1 expression in the stromal compartment of tumours derived from co-injection of HT29-M6 and Snail1 KO MEFs than in that of tumours in the control group (Supplementary Figure 3A). As shown in Figure 2A, tumours derived from co-injection of colon cells and Snail KO MEFs were significantly smaller than the tumours of the control group. Supporting these data, Ki67 immunostaining showed a significant proliferation index decrease in tumours from colon cells and Snail1 KO MEFs (Figure 2B). Interestingly, muscular

layer invasion was observed in 67% of tumours derived from co-injection of HT29-M6 and Snail1 wt MEFs, while no tumour from Snail1 KO MEFs showed muscular invasion (Figure 2C). In line with these data, down-regulation of E-cadherin was observed in tumours derived from colon cancer cells and Snail1 wt MEFs by immunohistochemistry (Supplementary Figure 3B). These results demonstrated that depletion of Snail from fibroblasts decreased their ability to support xenograft tumour growth and invasion and supported in-vitro Snail-derived effects.

To identify mediators involved in Snail1-dependent paracrine signalling between fibroblasts and colon cancer cells, we investigated whether Snail1 expression in fibroblasts modifies the production and release of cytokines, growth factors and other immuno-modulators. Thus, a human cytokine array with conditioned medium from 1.BR3.G-Snail1 transfected cells and 1.BR3.G-PCDNA3 as control was performed to analyze the protein concentration of 80 human secreted proteins. Four up-regulated (GRO-alpha, MCP-3, Fractalkine and I-309) and two down-regulated (IL-10 and G-CSF) cytokines were observed, indicating a cytokine change profile derived from Snail1 expression in fibroblasts (Figure 3A).

These data suggest that the Snail-dependent paracrine effect on colon cancer cells may involve cytokine profile changes. Therefore, to verify the effect of these cytokines, SW480-ADH-Mock cells were incubated with corresponding recombinant proteins. Preliminary results showed an increase in SW480-ADH-Mock cell migration when cells were incubated with Fractalkine or MCP3 recombinant proteins (Figure 3B). Similarly, a trend toward statistical increase of SW480-ADH-Mock cell migration was observed when incubation was with I-309 or GRO-alpha (Figure 3B).

Different approaches have attempted to validate further the association between Snail1 and cytokine expression. Thus, mRNA expression levels of Snail1 and target cytokines were analysed in 1) 8 CAFs with different SNAI1 expression levels; 2) Snail1 KO and wt MEFs; 3) 1.BR3.G-Snail1 transfected cells and 1.BR3.G-PCDNA3; 4) human colon tumour tissues from 65 patients. Interestingly, MCP3 and Snail1 expression levels are associated in all experimental approaches (Figure 3C-3F). Remaining cytokines, such as GRO-alpha and Fractalkine, also showed statistical association with Snail1 expression levels, but not under all conditions (data not shown). Taken together, these data support the view that, at least, changes in mRNA and protein expression levels of MCP3 are involved in the Snail1-dependent paracrine effect on colon cancer cells.

In summary, the clinical implication of this study lies in the observation that *SNAI1* expression is up-regulated in the stroma of human colon cancer patients. Moreover, in human colon tumour samples, *SNAI1* expression was associated with CAF markers, like  $\alpha$ -SMA and FAP, which have previously been related to colorectal cancer patient survival.<sup>18</sup> In addition, the overlapping immunostaining of SNAI1 and CAF markers in parallel sections of tumour samples support these data, which point to the expression of *SNAI1* as a CAF marker. The identification of new markers for progression in colon and other cancers is essential for understanding the cross-talk between fibroblasts and cancer cells, which would identify new targets for developing novel strategies to interfere with cancer progression.

Our data describe a novel role of Snail1-derived paracrine effects on cancer cells. Thus, Snail expression in fibroblasts increases the tumorigenic abilities of co-cultures of colon cancer cells by increasing cell migration and proliferation. Corroborative data were also obtained when Snail1 wild-type or knock-out MEFs were used. In addition,

data obtained from primary CAFs with similar results to results with cell lines indicate the relevance of *SNAIL* expression in clinical settings. The in-vivo value of these findings is emphasized by the finding that co-injection of tumour cells with Snail KO fibroblasts developed smaller tumours with a lower proliferation index and less muscular invasion than co-injection with wild-type fibroblasts did. In contrast to our results, Snail1 expression has been related to a retard in cell proliferation.<sup>21</sup> However, these studies were performed over-expressing Snail1 in epithelial cells, while our results analyse the relevance of Snail1 in fibroblasts and its effect on co-cultured colon cells, demonstrating a novel paracrine effects of Snail1-expressing fibroblasts.

Analysis should be extended to identify the fibroblast-derived mediators of Snail1-dependent stimulatory in-vitro effects. However, our results suggest that the paracrine effect of fibroblasts' Snail expression engages with cytokine profile changes. Indeed, the direct effect of some of these cytokines was assessed in the induction of colon tumour cell migration. Moreover, association between MCP3 and Snail1 mRNA expression levels was confirmed under different experimental conditions, which suggest that this cytokine might be a mediator of Snail1-dependent paracrine effects of fibroblasts on cancer cell migration and proliferation.

In brief, the conclusions of the present study demonstrate the importance of Snail1 expression in fibroblasts and CAFs, enhancing the pro-tumorigenic abilities of colon cancer cells, in a way that involves cytokine profile changes.

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## FIGURE LEGENDS

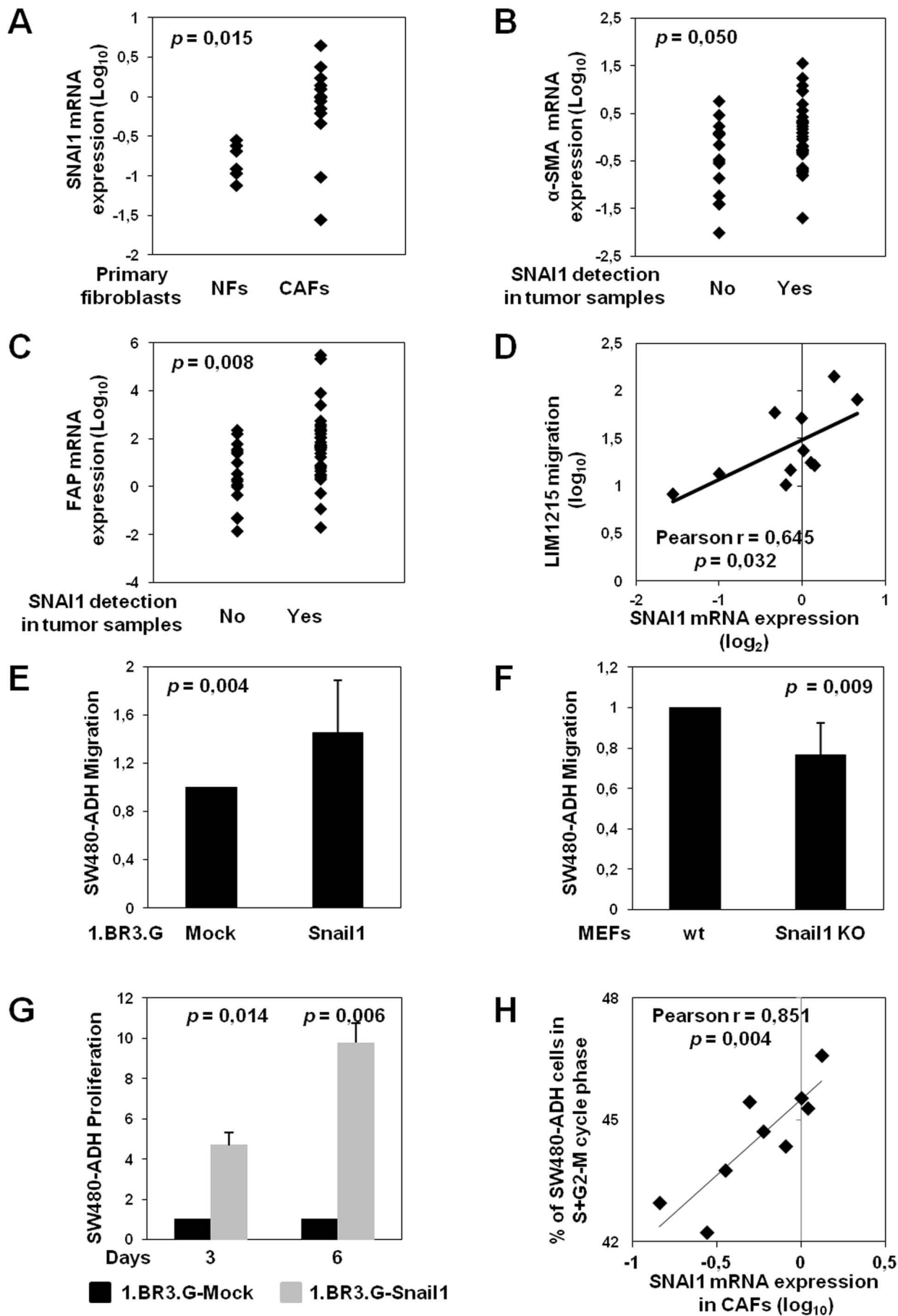
**Figure 1. *SNAIL* expression in human primary CAFs is suggested as an activated fibroblast marker and enhances colon cancer migration and proliferation in a paracrine manner.** Increased expression levels of *SNAIL* (A) in CAFs versus NFs. Association between *SNAIL* expression and  $\alpha$ -SMA (B) or FAP (C) gene expression levels in human tumour samples. Correlation between *SNAIL* expression levels of CAFs and migration of co-cultured LIM1215 colon cancer cells (D). Ectopic expression of *Snail1* in fibroblasts enhanced 48h migration of SW480-ADH-Mock colon cancer cells (E). SW480-ADH-Mock migration decreased when co-cultured with knock-out MEFs (F). Proliferation of SW480-ADH-Mock cells is stimulated by *Snail*-expressing fibroblasts (G). Correlation of *SNAIL* expression levels in CAFs from 9 different patients and the percentage of colon cells in phases S+G2-M after incubation with CAF-conditioned medium (H).

**Figure 2. *Snail1*-expressing fibroblasts are required for the co-adjuvant effect of these cells on colon cancer cell growth and invasion in co-xenografted nude mice.** Primary tumour growth was monitored for 32 days, showing a reduction of size in tumours derived from co-injection of tumour cells and *Snail1* KO MEFs (A). There were a significant proliferation index, measured by Ki67 immunostaining, and a decrease in tumours derived from colon cells and *Snail1* KO MEFs (B). Muscular layer invasion was observed only in tumours derived from co-injection of colon cells and wt MEFs (C).

**Figure 3. Association between MCP3 and *Snail1* expression levels in CAFs and human tumour tissues.** De-regulated proteins in conditioned medium from 1.BR3G-*Snail1* fibroblasts for Mock cells measured by Human Cytokine Array. (A) There was a

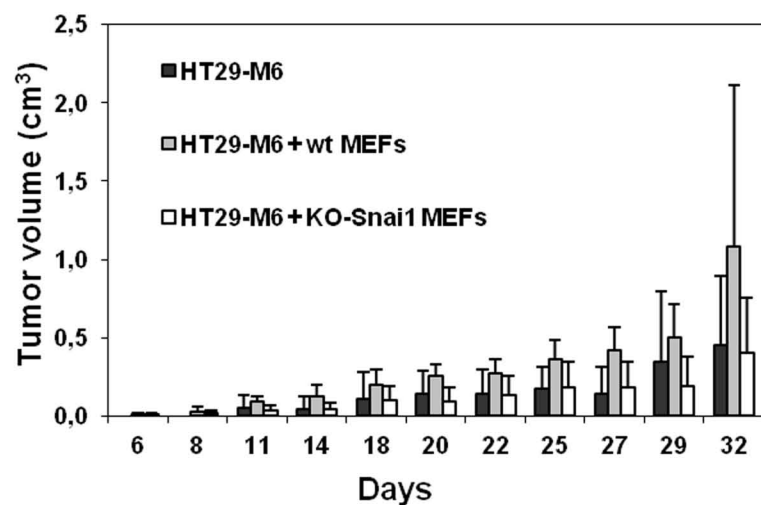
statistically significant increase in SW480-ADH-Mock tumour colon cell migration when cells were incubated with Fractalkine or MCP3 cytokines. Although there was no statistical difference, there was an increase of SW480-ADH-Mock cell migration when cells were incubated with I-309 and GRO- $\alpha$  cytokines (**B**). Association between MCP3 and SNAI1 mRNA expression levels in human primary CAFs (**C**), in Snail1 KO and wt MEFs treated with TGF- $\beta$  at different times (**D**), in 1.BR3.G-Snail1 transfected cells and 1.BR3.G-PCDNA3 (**E**) and in human colon tumour tissues from 65 patients (**F**).

# FIGURE 1

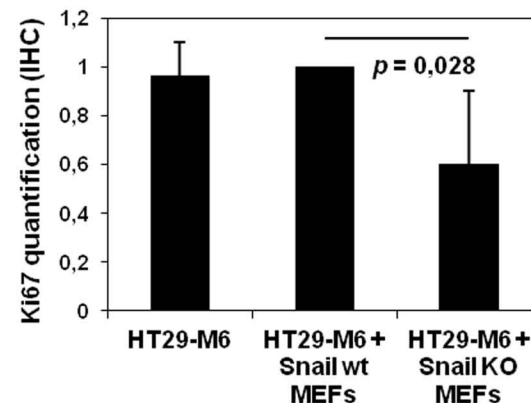
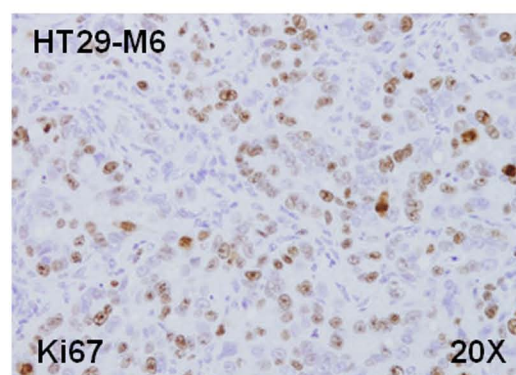
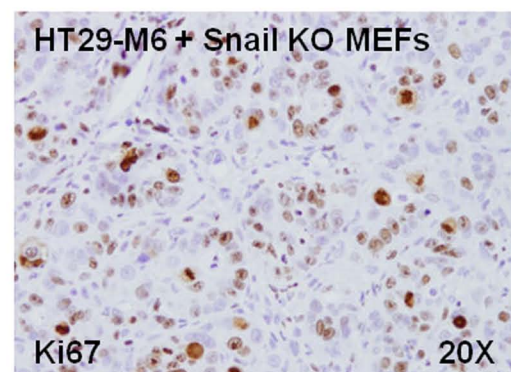
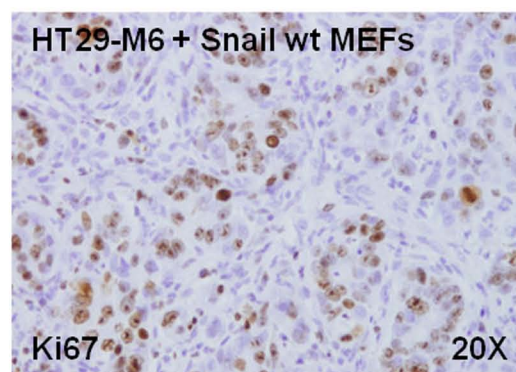


# FIGURE 2

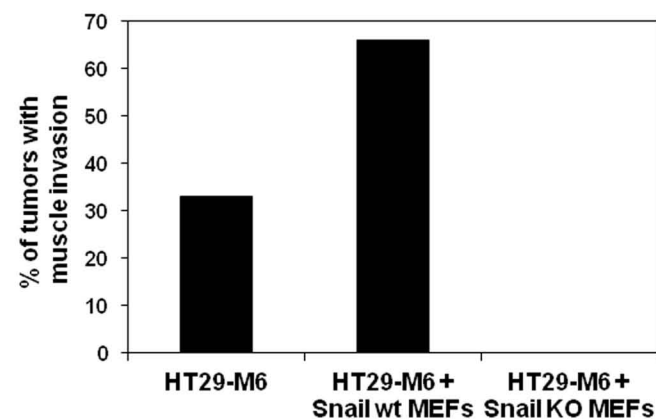
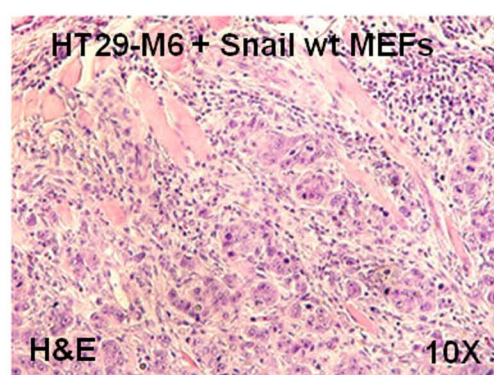
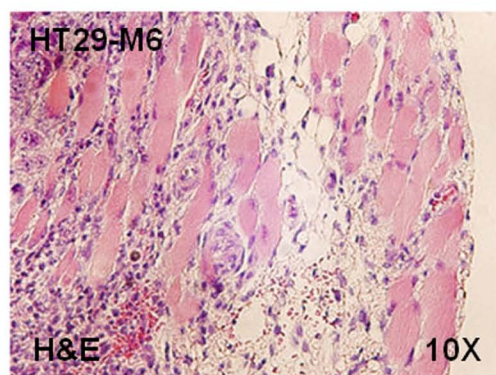
## A



## B



## C

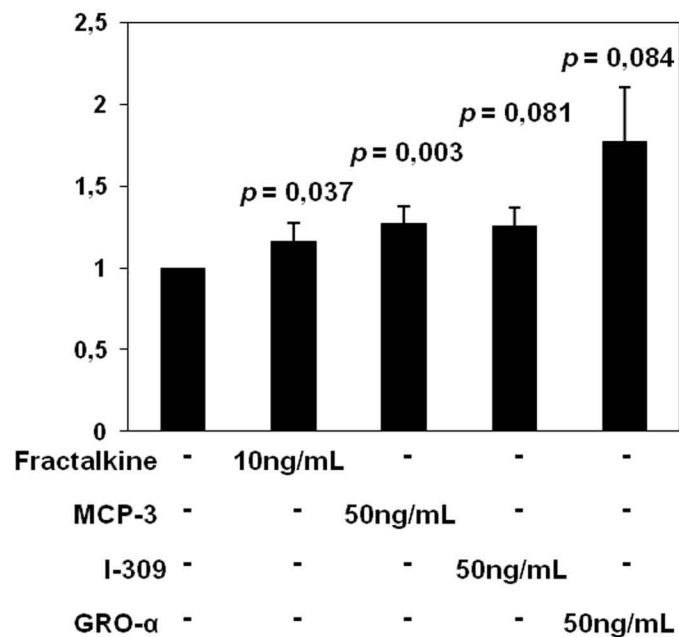


# FIGURE 3

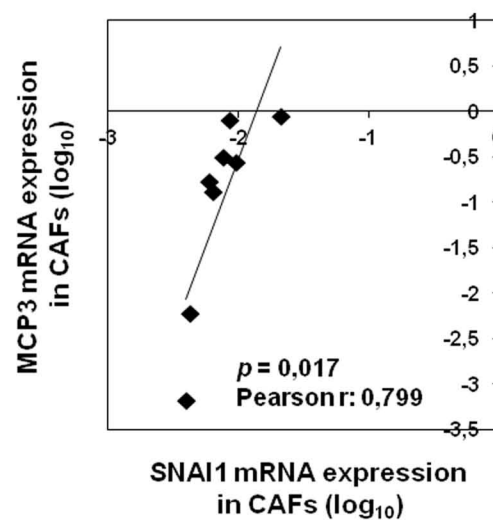
**A**

Cytokine	MEDIAN:		<i>p</i> value
	1.BR3G-SNAIL/ 1.BR3G-Mock	SD	
GRO-alpha	1,31	0,38	0,073
MCP-3	1,20	0,24	0,074
Fractalkine	1,17	0,08	<0,001
I-309	1,17	0,20	0,064
IL-10	0,92	0,10	0,079
GCSF	0,78	0,21	0,026

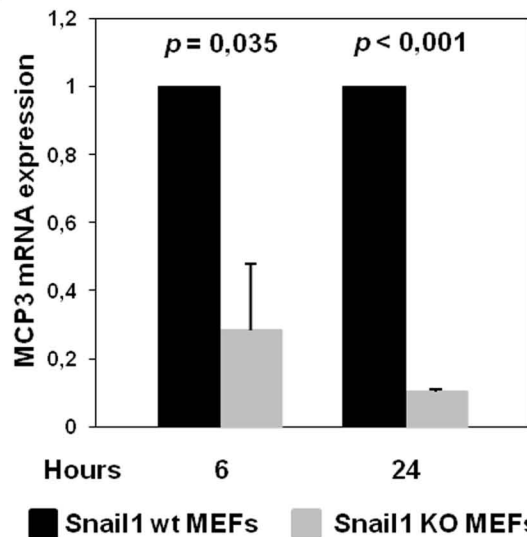
**B**



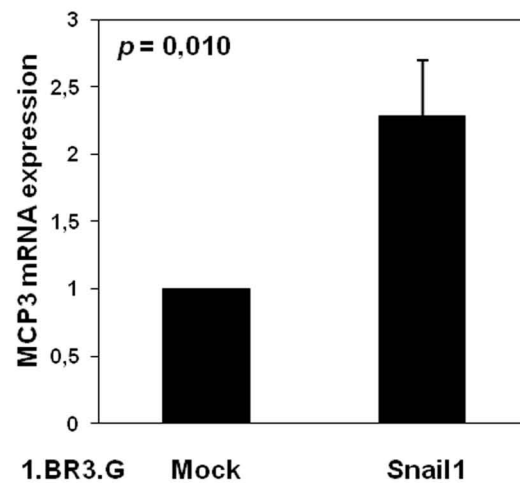
**C**



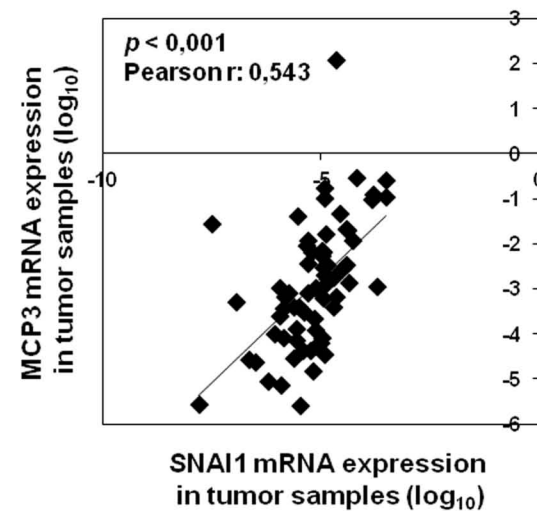
**D**



**E**



**F**



## **SUPPLEMENTARY INFORMATION**

### **Supplementary methodology**

#### *Establishment of primary colon cancer CAFs*

All the fibroblast strains were derived in the same way from explant cultures. Tissue samples from colon tumour were cut into small pieces approximately 2-3 mm<sup>3</sup> in size and seeded in foetal calf serum medium (FCS) with 200u/ml Penicillin, 200ug/ml Streptomycin, 100ug/ml Gentamicin and 2.5g/ml Amphotericin B. When outgrowths of fibroblasts appeared, the culture medium was replaced by FMB (Lonza) supplemented with FGM-2 Bulletkit (Lonza) to facilitate fibroblast growth. The remnants of the tissue were carefully washed away and CAFs were routinely maintained in FBM medium at 37°C in a humid atmosphere containing 5% CO<sub>2</sub>.

#### *Pan-cytokeratin and Vimentin immunofluorescence*

To determine CAF culture enrichment, Vimentin and Pan-cytokeratin were labelled by immunofluorescence.

Briefly, fibroblast cells cultured on pp4 plates were washed with cold phosphate-buffered saline (PBS) twice and then fixed with cold methanol for 30 sec. Following 5 washes with PBS (the last one with 0.05% Tween) at room temperature, fixed cells were incubated with specific primary antibody to Pan-cytokeratin antibody and Vimentin (monoclonal anti-pan cytokeratin, ab6401 Abcam; and monoclonal anti-vimentin, ab92547 Abcam, respectively) overnight at 4°C. Then, the samples were incubated for 1h with appropriate secondary species-labelled IgG Alexa-Fluor 488 or 546 (Invitrogen). After 4 PBS washes, slides were mounted with Vectashield Hard-Set mounting media with DAPI (4',6-diamidino-2-phenylindole, HCl) to view the nuclei



(Vector Laboratories Inc.) and were examined with a Nikon Eclipse Ti-S Epifluorescence microscope equipped with a DS-L2 digital camera (Nikon). Representative samples were taken from diverse areas of each cell culture to assess the percentage of positive cells.

#### *Culture cell lines*

Human colon cancer cell lines and MEFs were cultured in Dulbecco's modified Eagle medium (DMEM, Lonza), supplemented with 10% Fetal Bovine Serum (FBS), 2 mM L-glutamine, penicillin (100 units/ml) and streptomycin (100 ng/ml) at 37°C in a 5% CO<sub>2</sub> humidified atmosphere. 1.BR3.G immortalized human dermal fibroblasts were grown in EMEM (Lonza) with 20% FBS.

#### *Generation of Snai1 modified cell lines*

SW480-ADH and HT-29 human colon cancer cell lines stably expressing mouse Snai1 cDNA (SW480-ADH-Snai1 and HT-29-Snai1) or an empty vector (SW480-ADH Mock and HT-29 Mock) were generated by retrovirus-mediated gene transfer, as described earlier (Palmer et al. 2004). 1.BR3.G human dermal fibroblasts stably expressing mouse Snai1 cDNA were obtained by transfection, at 90% confluence, of 24 µg of either pcDNA3 SNAI1-HA or control pcDNA3 vector, using Lipofectamine reagent (Invitrogen), as recommended by the manufacturer. Then, medium was replaced with selective medium containing 1 mg/ml G418 (Sigma) for 3-4 weeks. Snai1 knock-out MEFs were generated as described in Batlle et al. (in press).

#### *Western blot analysis*

90 µg of cell protein lysate were used to analyse Snai1 protein expression by Western blotting with a mouse monoclonal antibody (Franci et al. 2006). β-actin was detected

with a rabbit polyclonal antibody (ab8227-50; Abcam). Cell protein was extracted with RIPA buffer (Sigma) and Halt Protease and Phosphatase Single-Use Inhibitor Cocktail (Thermo Scientific). 90 µg of protein from cell lysates were fractionated by 10% SDS-PAGE and blotted onto nitrocellulose membranes by the iBlot dry blotting transfer system (Invitrogen). Membranes were blocked and incubated with the corresponding antibodies as standard procedures. Quantification was done with the Odyssey Infrared Imaging System (Li-Cor Biosciences).  $\beta$ -actin was used to normalize gel bands.

#### *Co-culture of tumour colon cells and CAFs or fibroblasts for migration assays*

Colorectal tumour cell lines were co-cultured with colon primary CAFs or Snail-modified fibroblast on 8.0 µm-pore Transwells (Corning Incorporated Costar) for migration assays. The experiments were performed in Dulbecco's modified Eagle medium (DMEM; Gibco Life Technologies) containing 1% FCS media and penicillin/streptomycin antibiotics under standard conditions. Prior to co-culture, epithelial cells were labelled with a molecular probe (Cell Tracker Red CMTPX C34552, Invitrogen), in line with the manufacturer's instructions, to distinguish them from fibroblasts. Cells were co-cultured with physical separation, in a setting in which fibroblasts were seeded in the lower compartment of the transwell and the labelled colon cells in the upper one. After 48 h, epithelial cells that had reached the lower surface of the filter were recovered by trypsinization and counted by fluorescence with the microplate reader (Tecan Infinite 200Pro.) (excitation at 590nm; emission at 635nm) through interpolation using a standard curve.

#### *Conditioned medium harvest and cell cycle analysis by FACs.*

To obtain conditioned mediums, the standard medium, with 10% FBS, of Mock or Snai1 fibroblast cells as well as primary CAFs, was replaced by foetal bovine serum-free medium and incubated for 24 h. Afterwards, conditioned medium was harvested and centrifuged at 1,500 rpm for 6 min to remove cell debris. Mock cells were incubated with conditioned medium, diluted 1:4 in standard medium, for 24 h. Subsequently, Mock cells were detached from dishes with trypsin, fixed in 70% ethanol (-20°C) for 1 min and treated with 0.2 mg/ml RNase (Qiagen) and propidium iodide (Sigma Chemical Co.) at 20 µg/ml in PBS for 15 min at room temperature in darkness. Cell DNA content was evaluated in a MACSQuant Analyzer (Miltenyi Biotec). For computer analysis, using FACSDiva software (BD Biosciences), only signals from single cells were considered (20,000 cells/assay).

#### *Human protein cytokine array*

1.BR3.G-PCDNA3 and 1.BR3.G-Snail transfected cells (Lipofectamine 2000, Invitrogen) were cultured at 37°C in an atmosphere of 5% CO<sub>2</sub> for 24 h after transfection in DMEM with 10% FBS. Conditioned medium, harvested after 24h incubation in DMEM FBS-free medium, was centrifuged at 1,500 g to remove cell debris and filtered. Conditioned medium from each transient cell type was prepared and incubated with membranes containing an array of 80 human cytokine antibodies (RayBio® Human Cytokine Antibody Array 5, RayBiotech Inc.). Procedures carried out followed the manufacturer's instructions. The bound complex was detected with the Odyssey Infrared Imaging System (Li-Cor) at 800 nm, using Streptavidin DyLight 800 Conjugates (Rockland Immunochemicals). The experiment was repeated six times and cytokine deregulation was assessed by significant statistical differences.

#### *Statistical analysis*

Cell migration and proliferation assays, as well as comparison between mRNA *SNAIL* levels in NFs vs CAFs, were contrasted using the student's t-test. Correlation of *SNAIL* expression and the induction of colon cell migration or proliferation by primary CAFs were analyzed by Pearson's test. *SNAIL*,  $\alpha$ -*SMA* and *FAP* mRNA expression levels were analysed as described by Peña et al, 2005 and 2009, and their associations were analysed by the Student's t-test. *SNAIL* and cytokine mRNA expression levels in human tumour tissues or human primary CAFs were analysed as described by Herrera et al, 2013, and their associations were analysed by the Pearson correlation test. In 1.BR3G and Snail1 KO or wt MEFs, this association was also analysed by the Pearson test.

In all statistical tests, two-tailed P-values  $\leq 0.05$  were considered statistically different. All statistical results are derived from 2-4 independent experiments, each performed in duplicate.

Statistical analysis used the SPSS statistical package, version 14.0.

## **Supplementary Figure legends**

**Supplementary Figure 1.** The absence of epithelial cells in the established primary cultures was confirmed by PanCK and Vimentin immunostaining. LIM1215 colon cells were used as positive control for PanCK immunostaining (A). Increased expression levels of  $\alpha$ -SMA, a typical marker of activated fibroblasts, in CAFs versus NFs (B). Immunostaining overlapped between SNAIL1,  $\alpha$ -SMA and FAP in human colon tumour samples (C). Correlation between *SNAIL1* expression levels of CAFs and migration of co-cultured LIM1215 or SW480-ADH colon cancer cell migration (D).

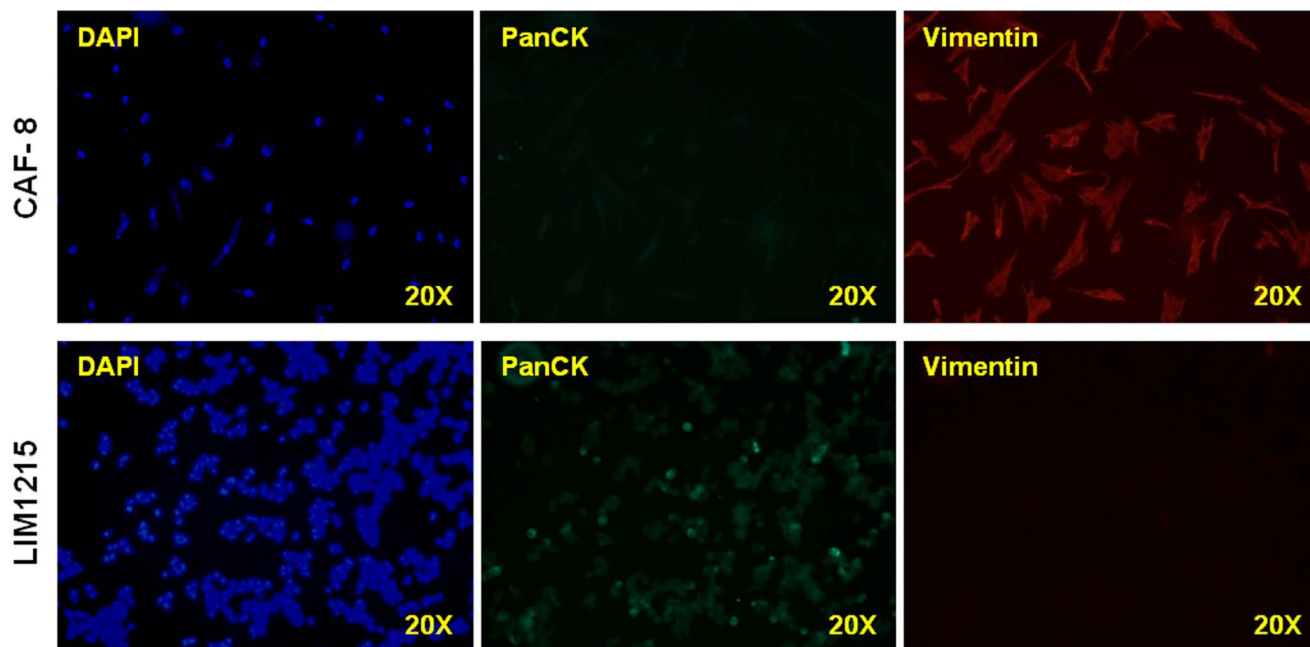
**Supplementary Figure 2.** **The increase in migration and proliferation of colon tumours is also observed when Snail1 is expressed by colon cells.** Western blot analysis of SW480-ADH and 1.BR3G Snail1 transfected cells, as well as Snail1 ko and wt MEFs (A). Ectopic expression of Snail1 in fibroblasts enhanced 48h migration of LIM1215 colon cancer cells (B). HT29-M6 (C) or LIM1215 (D) migration decreased when co-cultured with knock-out MEFs. 48h migration of SW480-ADH-Mock colon cancer cells co-cultured with SW480-ADH-Mock and SW480-ADH-Snail1 (E) or with HT-29-Mock and HT-29-Snail1 (F). Proliferation levels at 3 and 6 days of SW480-ADH-Mock colon cancer cells co-cultured with SW480-ADH-Mock or SW480-ADH-Snail1 (G). FACS analysis of the cell cycle of colon cancer cells showed an increase in S+G2-M phase cells after incubation of cells with conditioned medium from Snail1-expressing fibroblasts (H).

**Supplementary Figure 3.** **Snail1-expressing fibroblasts are required for the co-adjuvant effect of these cells on colon cancer cell invasion in co-xenografted nude mice.** Lower Snail1 expression is observed in the stromal compartment of tumours derived from co-injection of HT29-M6 and Snail1 KO MEFs than of tumours in the

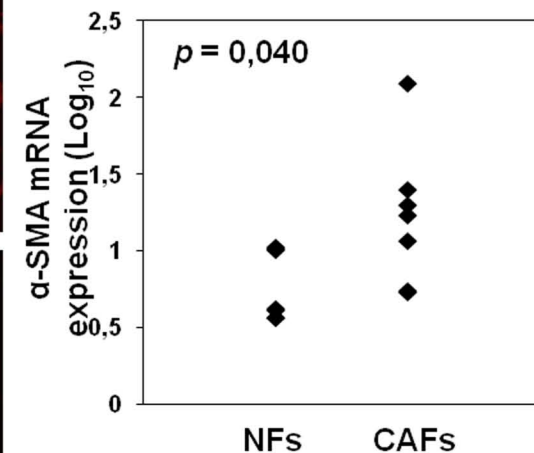
control group (A). E-cadherin immunohistochemistry shows protein down-regulation in tumours derived from colon cells and Snail KO MEFs.

# SUPPLEMENTARY FIGURE 1

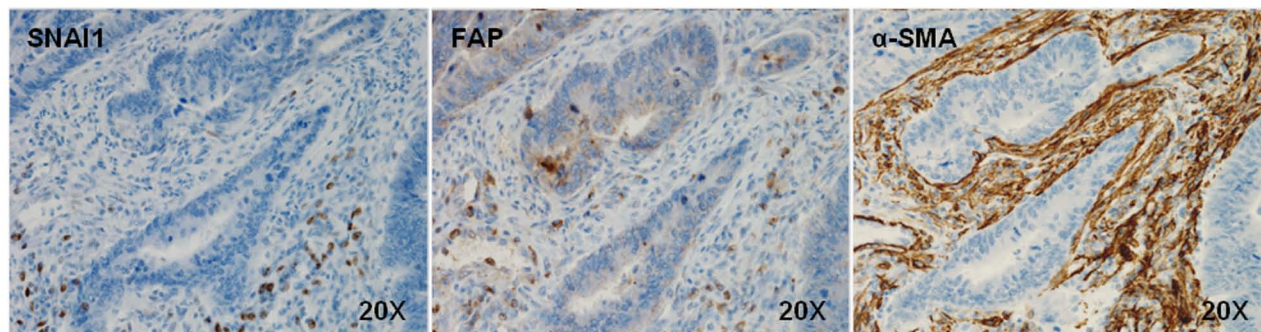
**A**



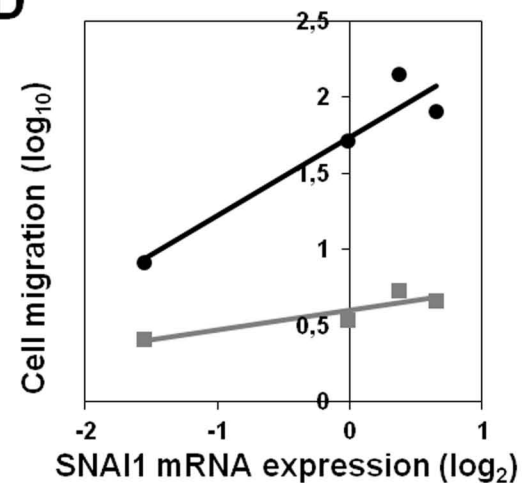
**B**



**C**



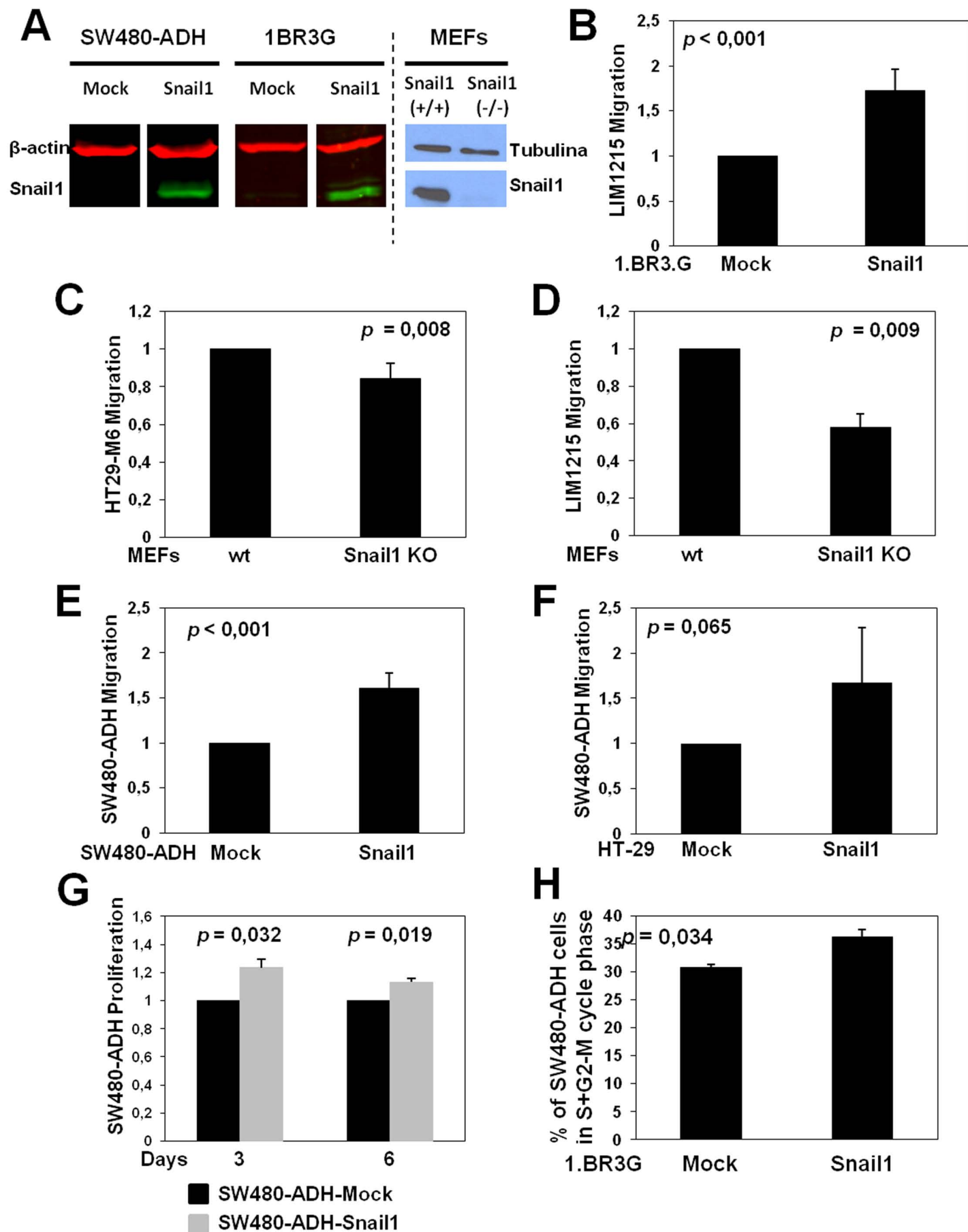
**D**



● LIM1215: Pearson  $r = 0,953$   
 $p = 0,047$

■ SW480-ADH: Pearson  $r = 0,906$   
 $p = 0,094$

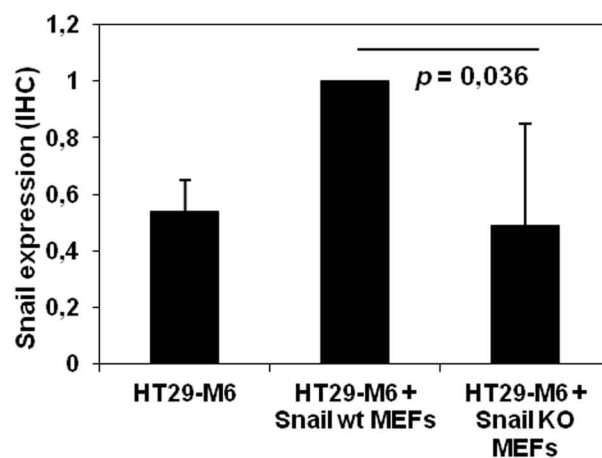
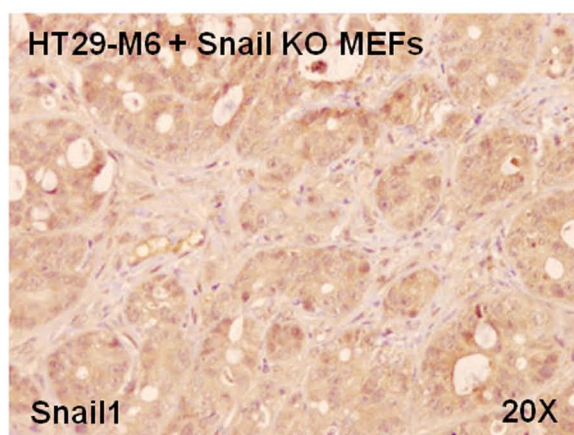
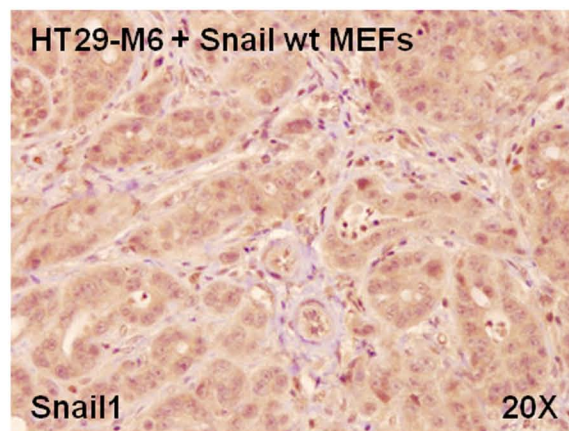
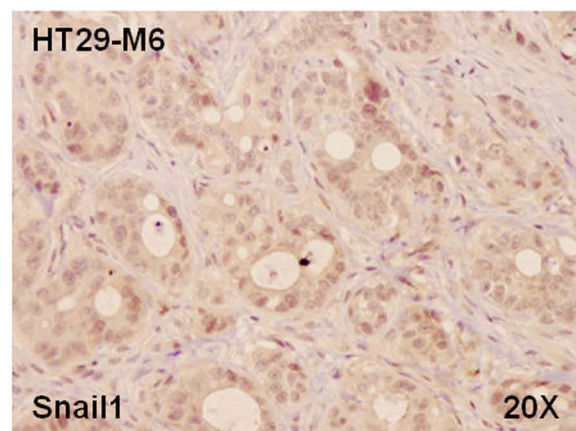
# SUPPLEMENTARY FIGURE 2





# SUPPLEMENTARY FIGURE 3

## A



## B

