Snail1 controls cancer-associated fibroblast action on tumor cell invasion and metastasis

Lorena Alba-Castellón[1], Rubén Olivera[1], Aida Mestre[1], Raúl Peña[1], Mercedes Herrera[2], Félix Bonilla[3], Josep Baulida[1], J. Ignacio Casal[4], Cristina Peña[2], and Antonio García de Herreros[1,5,6]

[1] Programa de Recerca en Càncer, IMIM-Hospital del Mar, 08003 Barcelona, Spain

[2] Servicio de Oncologia Médica, Hospital Puerta de Hierro, 28222 Majadahonda, Spain

[3] Centro de Estudios Biosanitarios, Madrid, Spain

[4] Centro de Investigaciones Biológicas, CSIC, 28040, Madrid, Spain

[5] Departament de Ciències Experimentals i de la Salut, Universitat Pompeu Fabra, 08003 Barcelona, Spain

[6] To whom correspondence should be addressed at Programa de Recerca en Càncer, IMIM, Parc de Recerca Biomèdica de Barcelona, C/ Doctor Aiguader 88; 08003 Barcelona, Spain; e-mail: agarcia@imim.es.

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SUMMARY

Snail1 transcriptional factor is essential for triggering epithelial-to-mesenchymal transition (EMT) and inducing tumor cell invasion. We report here an additional, EMT-independent action of Snail1 on tumor invasion: its expression in cancer-associated fibroblasts is necessary for enhancement by these cells on epithelial cells tumor invasion. Snail1 expression in fibroblast requires signals derived from tumor cells such as TGF-β; reciprocally, in fibroblasts Snail1 organizes a complex program that favors collective invasion of epithelial cells at least in part by the secretion of diffusible signaling molecules, such as prostaglandin E_2. The capability of human or murine tumor-derived cancer associated fibroblasts to promote tumor invasion is associated to Snail1 expression and obliterated by Snail1 depletion. In vivo experiments show that Snail1 depletion in mice prevents the invasion of breast tumors and epithelial tumor cells co-xenografted with Snail1-depleted fibroblasts originate tumors with lower invasion than those transplanted with control fibroblasts. Therefore, these results demonstrate that the role of Snail1 in tumor invasion is not limited to EMT but dependent on its expression in stromal fibroblasts where it orchestrates its activation and the crosstalk with epithelial tumor cells. Moreover, they point to the interference of Snail expression as a promising target for preventing the action of stromal fibroblasts on tumor progression.

INTRODUCTION

Epithelial to mesenchymal transition is a process characterized by the loss of epithelial features and the gain of mesenchymal traits. This process widely described during embryo development provides the cells with higher migration, besides inducing other cancer hallmarks such as resistance to apoptotic insults or unlimited replication. As consequence cells that have undergone an EMT present a higher capability to invade both *in vitro* and *in vivo* (Thiery et al, 2009). However, the precise contribution of EMT to tumor invasion *in vivo* is still a matter of discussion since the number of tumor cells displaying mesenchymal characteristics is low in human tumors (Tarin et al, 2005). In many cases tumor cells exhibit a collective invasion maintaining epithelial characteristics (Friedl and Alexander, 2011).

Molecularly EMT is associated with the down-regulation of E-cadherin (*CDH1*) gene expression and the up-regulation of mesenchymal markers (Thiery et al., 2009; Garcia de Herreros and Baulida, 2012). Among the several *CDH1* transcriptional repressors induced during EMT Snail1 has received a particular attention since is the first one to be temporally induced and is required for the induction of other *CDH1* repressors, such as Zeb1/ 2; consequently; Snail1 transfection promotes an extensive EMT (Garcia de Herreros and Baulida, 2012). Snail1 is a transcriptional factor that recruits to *CDH1* promoter co-repressors such as histone deacetylases 1 and 2 (Peinado et al., 2004), Polycomb repressive complex 2 and G9a histone 3 lysine methylases (Herranz et al, 2008; Dong et al, 2012) and PRMT5 histone 4 arginine demethylase (Hou et al, 2008). However, Snail1 does not act uniquely as repressor since during EMT it is also present in the promoters of activated mesenchymal genes such as fibronectin (Stanisavljevic et al, 2011). A recent report demonstrates that the activity of Snail1 as repressor or

activator relies in its acetylation in lysines 146 and 187 by CREB-binding protein (Hsu et al, 2014) since the modification of these residues promotes the Snail1 switch from epithelial-repressed to mesenchymal-induced genes. In accordance with this function in the activation of mesenchymal genes, Snail1 action is not limited to EMT. Besides other effects in embryonic stem cells (Lin et al, 2014), Snail1 is required for a complete fibroblast response to PDGF or TGF-β since signaling by these factors is severely affected by Snail1 genetic depletion (Rowe et al, 2009; Batlle et al, 2013).

It is now totally accepted that tumoral cells modify their context creating a micronvironment, the tumor stroma that provides signals required for the acquisition of many cancer hallmarks (Pietras and Ostman, 2010). Among the components of the tumor estroma, the cancer-associated fibroblasts (CAFs) have received special attention due their capability to support growth and invasion of epithelial cells (Gaggioli et al. 2007; Li et al, 2012; Calon et al., 2012; Herrera et al, 2014). When analyzing Snail1 expression in tumors we have detected that is mainly present in fibroblasts in the tumor stroma of breast and colon tumors (Franci et al., 2009; Stanisavljevic et al, 2015) and correlates with a bad prognosis even in low-grade tumors. These studies agree with recent reports indicating that stromal fibroblasts markers are the best indicators of prognosis for colon neoplasms, even in low-grade tumors (Calon et al, 2015). In this article we have analyzed the cooperation between epithelial and stromal cells during tumor invasion. Our results show that the stimulation of epithelial cell invasion by mesenchymal cells requires the Snail1-dependent activation of these fibroblasts by cytokines released by tumor cells. Activated fibroblast guide the process of invasion producing factors, such as prostaglandin E_2 (PGE_2), capable to cause the collective migration of epithelial cells.

RESULTS

Snail1 expression in fibroblasts is required for induction of epithelial cell invasion

As first step to set our cellular model, we analyzed the invasiveness of a panel of breast or colon tumor cell lines in Boyden Chambers (Fig S1A). From this panel we selected a representative line from both breast or colon showing low (MCF-7 and HT-29 M6) or high (MDA-MB231 and SW-620) invasion. We analyzed the cooperation between cell lines displaying low invasiveness and mesenchymal cells. As cellular models of fibroblastic cells we used murine embryo fibroblasts (MEFs) and mesenchymal stem cells (MSCs). These cells have been shown to contribute to the formation of cancer-associated fibroblasts in the tumor stroma and promote growth and progression of tumors (Karnoub et al, 2007; Mishra et al, 2008; Quante et al, 2011). In order to specifically analyze the invasion of HT-29 M6, these cells were stably transfected with RFP and only cells showing red fluorescence in the lower part for the membrane were considered. As shown in Figs 1A and B, co-seeding of HT-29 M6 with MSC increased the number of epithelial cells migrating through the matrix. This effect was observed both on Matrigel (Figs 1A and B) and Collagen1 (Fig S2A) matrices and was inhibited by a general metalloprotease inhibitor, GM6001 (GM) (Galardy et al, 1994) (Figs S2A and S2B). The stimulation of invasion required a lower number of MSCs than epithelial cells: a ratio of 1/10 of MSC with respect to HT-29 M6 cells produced a close to maximal effect (Fig S2C). Interestingly MSC deficient in Snail1 (Fig S3A) were unable to stimulate epithelial invasion either through Matrigel (Figs 1A and B) or Collagen 1 (Fig S2A).

These results were also repeated using MEFs either wild-type or depleted in Snail1 (Fig S3A). Only wild-type cells enhanced HT-29 M6 invasion (Fig 1C); Snail1 KO cells did not modify this parameter.

We have previously shown that Snail1-depletion in MSCs greatly compromises their response to TGF-β (Batlle et al, 2013). Although the activation of its receptor and early responses are not affected, Snail1-depletion prevents later responses to TGF-β (Batlle et al, 2013). Therefore, we determined if the enhancement in HT-29 M6 invasion produced by MSCs was dependent on signaling by this cytokine. As shown in Fig 1B, the broadly used TGF-β receptor I (TBR) inhibitor SB505124 (SB) (daCosta Byfield et al, 2004) totally prevented the action of MSCs on HT-29 M6 invasion, although it did not significantly affect basal HT-29 M6 invasion. The release of the cytokine to the cell medium was also determined. HT-29 M6 secreted considerably more TGF-β than MSCs (Fig 1D); no significant differences were observed between the amounts produced by wild-type or Snail1 KO cells in these conditions. The co-culture of epithelial and mesenchymal cells only showed an additive effect. Therefore, these results indicate that in these experiments TGF-β is produced mainly by the tumor cells.

We also determined the effect of TGF–β and HT-29 M6 on MSCs invasion. Following an experimental approach similar to that described in Fig 1A, MSCs were transfected with a GFP-expressing plasmid to distinguish them from HT-29 M6. As shown in Fig 1E (right), exogenous TGF-β remarkably increased MSCs invasion. As expected this effect was blocked by addition of the TBR inhibitor SB. An even greater stimulation was also observed when HT-29 M6 cells were included in the co-culture; this up-regulation was sensitive to the TBR inhibitor SB (Fig 1E, left). Neither TGF-β nor HT-29 M6

stimulated the migration of Snail1-depleted MSCs (Fig 1E) indicating that Snail1 is required for a complete response to TGF-β.

We analyzed the morphological characteristics of the invading co-culture, using unlabelled HT-29 M6 and MSCs expressing GFP. As shown in Fig 2, GFP-labelled MSCs were observed below HT-29 M6, preceding them and likely leading their migration. Epithelial cells invaded collectively although we detected a small change in the morphology of the cells placed in the interface; they adopted a more elongated phenotype with a basal nuclei opposed to the area of migration. Mesenchymal cells exhibited a stretched morphology (Figs 2). Invading HT-29 M6 cells did not show any sign of EMT; they did not express Snail1 that was restricted to MSCs, and maintained the expression of E-cadherin (Fig 2). Moreover, we did not observe any mesenchymal putatively generated from HT-29 M6 since all contained GFP.

Differently to wild-type cells, MSCs deficient in Snail1 did not invade and remained much more associated to the epithelial layer (Fig 2). A similar morphology was observed when HT-29 M6 were co-cultured with wild-type MSCs in the presence of the TBR inhibitor SB, in accordance with previous results indicating that fibroblast activation by TGF-β is deficient in Snail1-depleted cells. As expected this inhibitor, decreased the levels of P-Smad2, used as a surrogate marker of TGF-β signaling, in both mesenchymal and epithelial cells. This staining also indicated that MSCs were indeed binding TGF-β.

In order to ascertain the signals triggered by the TGF-β/Snail1 axis in MSCs that might be involved in HT-29 M6 invasion, we investigated the genes stimulated differently by this cytokine TGF-β in MSCs wild-type versus Snail1 KO. We compared the global

transcriptomes of these two cells lines after incubation with TGF-β; 803 genes that were regulated by this cytokine in control MSCs showed different levels in TGF-β-stimulated Snail1 KO cells (see the full list in GEO repository; accession number GSE74058). Among these genes, we selected those genes categorized in cell-cell communication, growth factors or extracellular matrix degradation; they are presented in Fig S4A. As shown, several metalloproteases, protease inhibitors and growth factors were differently expressed. Besides these, we also observed differences in *Ptgs2* (prostaglandin-endoperoxidase synthase 2) also known as cycloxigenase 2 or Cox2, an enzyme required for the synthesis of prostaglandins including prostaglandin E_2 (PGE_2), a molecule that regulates tumor cell proliferation, invasion, apoptosis and angiogenesis (Wang and DuBois, 2010).

We also determined if this time of treatment with TGF-β indeed mimicked the effect of co-culture with HT-29 M6, and the levels of these RNAs were stimulated in MSCs by HT-29 M6 and altered by Snail1 depletion. We took advantage of the different origin of MSCs, murine, and HT-29 M6, human. Expression of *Snail1*, *Mmp13*, *Pdgfb* and *Cox2* was significantly increased in MSCs when HT-29 M6 cells were also co-cultured (Fig S4B); the RNA corresponding to another gene involved in PGE_2, *Ptges2* was not increased. We also determined the levels of another enzyme controlling PGE_2, 15-hidroxi prostaglandin dehydrogenase (15-Hpgd) that is negatively controlled by Snail1 (Mann et al, 2006). *15-Hpgd* RNA levels were substantially decreased by co-culture of with MSCs with HT-29 M6 (Fig S4B). The mRNAs corresponding to *Snail1*, *Mmp13* and *Cox2* were significantly down-regulated in MSCs KO for Snail1 with respect to wild-type MSCs when these cells were co-cultured with HT-29 M6; on the contrary, *15-*

Hpgd was markedly increased (Fig S4B). Levels of *Ptges2* and *Pdgb* were not different.

PGE_2 secretion by fibroblast regulates epithelial cell invasion

Since the mRNAs corresponding to two key enzymes controlling PGE_2 were altered in Snail1 KO cells, we further investigated the relevance of this molecule. First, the levels of PGE_2 in the cellular medium were determined. Addition of TGF-β increased the secretion of this prostaglandin by wild-type but not Snail1-deficient MSCs (Fig 3A). HT-29 M6 only produce very limited amounts of the cytokine; however, co-culture of both cells greatly increased the amount of PGE2 present in the culture medium, an up-regulation that was sensitive to the TBR inhibitor SB and to the expression of Snail1 in MSCs (Fig 3A). Addition of the Cox-2 inhibitor Celecoxib (Penning et al, 1997) also remarkably decreased the secretion of PGE_2 to the cell co-culture medium (Fig 3A). Therefore, we concluded that HT-29 M6-produced TGF-β induces PGE_2 secretion by MSCs in a Snail1-dependent manner.

We also examined the relevance of PGE_2 production by MSCs for HT-29 M6 invasion. As shown in Fig 3B, Celecoxib addition reversed the MSCs enhancement of HT-29 M6 invasion. We also used two other compounds that specifically block PGE_2 receptors EP2 and EP4, and also Crenolanib, a PDGF antagonist. Addition of the EP4 receptor inhibitor L-161,982 (L-161) (Cherukuri et al, 2007) prevented the effect of MSCs on HT-29 M6 cells; on the contrary, the EP2 receptor inhibitor PF04418949 (PF-044) (af Forselles et al, 2011) or Crenolanib (Dai et al, 2013) did not reverse the stimulation. L-161 also prevented the stimulation in HT-29 M6 invasion detected when supplementing

these cells in the lower chamber of a Boyden Chamber with conditional medium of wild-type MSCs (Fig S5A).

In accordance with these results, PGE_2 stimulated HT-29 M6 invasion even in the absence of MSCs (Fig 3C); this effect was prevented by addition of L-161 but not of PF-044, further indicating that PGE_2 signals through the EP4 receptor in HT-29 M6 cells. Stimulation of invasion by the prostaglandin was dependent on its addition in the lower compartment of the Boyden Chamber, suggesting that it was working as a chemo-attractant; addition of the same concentration of PGE_2 in the upper chamber did not enhance invasion (Fig S5B). As expected, PGE_2-induced invasion was dependent on the action of metalloproteases since it was blocked by a general inhibitor of these enzymes (Fig S5B). PDGF-BB addition also increased HT-29 M6 invasion, a stimulation that was prevented by Crenolanib (Fig 3C). The effect of this inhibitor preventing the action of PDGF but not of MSCs suggests that this factor is not contributing to the stimulation by MScs of HT-29 M6 invasion.

Since inhibition of PGE_2 action promoted a remarkable action on HT-29 M6 invasion in co-cultures we tried to rescue the lack of stimulation of MSCs KO by the supplementation of PGE_2. As seen in Figs 3C, PGE_2 increased HT-29 M6 invasion in co-cultures with MSC (Snail1 KO) although not as much as when directly added to the epithelial cells.

The effect of the inhibitors and PGE_2 was also determined on the invasion of MSCs. As shown in Fig 3D, Celecoxib, L-161 or Crenolanib did not prevent the up-regulation in MSCs invasion caused by co-culture with HT-29 M6. Neither PGE_2 nor PDGF-BB increased MSCs invasion when added directly to these cells (Fig 3E). In accordance with the different effects of PGE_2 on HT-29 M6 and MSC, the analysis of the

morphology of the co-cultures showed that L-161 did not prevented the migration of MSC but it impaired that of HT-29 M6 (Fig S6). Consequently, MSC moved away from the epithelial cells that did not follow them.

We also characterized the invasion of HT-29 M6 promoted by PGE_2 addition analyzing the RNAs modified by this treatment. Among the 2,190 genes differently expressed (see the full list in GEO repository; accession number GSE74058), we detected several proteases and factors involved in cell-cell communication (Fig S7A). The up-regulation in *MMP3* and was verified by RT-PCR (Fig S7B); however, we did not detect any significant change in *COX2* and *CDH1* and only a slight increase in *SNAIL1* that was not confirmed by Western blot (Fig S7C). 15-Hpgd protein was not modified either. The morphology of invading cells was also determined upon PGE_2 addition. As observed, no phenotypic differences were observed in the cells that invade collectively (Fig S7D).

The reproducibility of the MSC effects on epithelial cell invasion in other cell lines was also determined. As seen in Fig S8A-D, invasion of intestinal HCT-116 or breast MCF-7, T47D and SK-BR3 cells was higher in the presence of wild-type MSCs; Snail1 null MSCs showed a lower enhancement of this parameter. In all these cases, the effect of MSCs was significantly (HCT-116, SK-BR3) or totally (T47D, MCF-7) prevented by addition of Celecoxib or L-161 (Fig S8A-D), indicating that it involved PGE_2 production and signaling. Accordingly, invasion of the four cell lines was stimulated by addition of PGE_2 (Fig S8E). Curiously, not all the cells showed the same sensitivity to TBR inhibitor: contrary to the other cell lines, enhancement of MCF-7 invasion by MSCs was not affected by addition of SB (Fig S8C). This result suggests that stimulation of MSCs by MCF-7 was not dependent on the action of TGF-β.

In accordance with the previous results MSCs invasion was up-regulated by co-culture by all the epithelial cells tested, thus HCT-116, T47D and MCF-7 (Fig S8F). Different to the other two cell lines, that were significantly sensitive to TBR inhibition, MCF-7 stimulation of MSCs invasion was only very slightly affected by the addition of SB.

Stimulation of tumor cell invasion by cancer-associated fibroblasts is also dependent on Snail1 expression

We also determined if the requirement for Snail1 expression was also observed with fibroblasts derived from epithelial tumors. In order to perform this study we generated tumors in murine model of breast cancer expressing polyoma middle T antigen under the control of *MMTV* promoter (MMTV-PyMT). Fibroblasts were obtained from these tumors as reported in Methods (Herrera et al, 2013). Since these animals also hold floxed and deleted *Snail1* genes they were transfected with Cre recombinase to eliminate Snail1 expression. We chose two cancer-fibroblasts lines, CAF-1857 and 52149, with high Snail1 expression that were depleted in *Snail1* RNA upon Cre recombinase transfection (Fig 4A). They were co-cultured with a cell line also generated from breast, MCF-7 cells. Both Snail1-depleted CAFs populations showed altered PGE_2 metabolism with respect to the control since in one case *Cox2* was down-regulated (CAF-52149) whereas the other one (CAF-1857) presented increased levels of 15-*Hpgd* (Fig 4A). Both wild-type CAFs populations significantly enhanced MCF-7 invasion; this stimulation was abolished by Celecoxib and L-161 indicating that it requires PGE_2 synthesis and action, and was not observed in the Snail1-depleted CAF populations (Fig 4B).

Other results obtained with fibroblasts derived from human tumors also showed an association between Snail1 levels and invasion. Different populations of colon tumors-derived fibroblast were used in these assays (Herrera et al., 2013, 2014). Snail1 expression was analyzed and CAFs were classified as Snail1-high (those showing an expression greater than 66% of the average) or Snail1-low (lower than 33% of the average). *Cox2* RNA expression correlated with that of *Snail1* in these cells (Fig. 4C). The capability to stimulate HT-29 M6 invasion was also associated to Snail1 expression since it was higher for Snail1-high than Snail-low populations. Moreover, it was sensitive to the addition of SB or Celecoxib to the culture medium (Fig. 4D). Finally, Snail1-high CAFs also showed a higher stimulation of PGE_2 production by co-culture with HT-29 M6 cells than Snal1-low CAFs, a stimulation that was sensitive to SB (Fig. 4E).

Expression of Snail1 in mesenchymal cells is required for fibroblast-dependent enhancement of epithelial tumor invasion in vivo

In order to verify the relevance of Snail1 expression in fibroblasts we use mentioned murine model of breast cancer by expression of PyMT (MMTV-PyMT). We generated murine lines carrying this oncogene, deleted or floxed alleles of *Snail1* (also a wild-type allele as control) and a Cre recombinase-Estrogen Receptor (Cre-ER) fusion protein under the control of the ubiquitous *β-Actin* promoter (β-Actin-Cre-ER). In this mice, Cre-ER was activated and, therefore, Snail1 depleted, by injection of tamoxifen when animals where eight weeks-old. Control animals (MMTV-PyMT, β-Actin-Cre-ER, Snail1+/Flox) developed tumors earlier than mice depleted in Snail1 and presented a

lower survival (Fig 5A). Eight weeks after tamoxifen injection in both animal lines tumor burden was higher in Snail1 WT than Snail1 KO mice (Fig 5B).

Animals were euthanized in both cases when tumors reach a pre-specified size and tumors were analyzed. As shown in Fig 5C, Snail1 WT but not Snail1 KO mice showed Snail1 expression in the nuclei of stromal cells, apparently fibroblasts localized in areas of invasion. Snail1 was never detected in tumor epithelial cells. The morphology of the tumors was different and Snail1 KO mice originated mostly adenomas, with compact glandules and with low presence of stromal cells (Fig 5D). On the contrary, Snail1 WT animals developed tumors with features of late carcinomas and with an abundant stroma. Snail1 expression also controlled the invasion to lymph nodes, determined analyzing the presence of CK19-positive colonies (Figs 5E-F). The percentage of mice presenting epithelial cells in the lymph nodes was dependent on Snail1 expression since was much higher in Snail1 WT than in Snail1 KO mice.

To more specifically evaluate the contribution of stromal Snail1 in invasion we used an orthotopic implantation model. Epithelial tumor cells were isolated from PyMT tumors (ePyMT). *In vitro* invasion of these cells was also stimulated by co-culture with MSCs (Fig S9); this stimulation was inhibited by Celecoxib or SB. As above, Snail1-depleted MSCs only very slightly increased ePyMT invasion (Fig S9). ePyMT cells alone or with MSCs, either Snail1 WT or KO, were allografted in the mammary fat pad of SCID mice. No differences were observed in the growth or volume of the tumors arising in the three conditions (Fig 6A). As expected, tumors obtained using control MSCs showed Snail1 presence in stromal fibroblasts whereas those from Snail1 KO did not (Fig 6B). The morphology of these tumors was also different since Snail1 WT tumors presented features of an advanced carcinoma, with disorganized epithelial structures and nuclei

of different sizes and shapes (Fig 6C). On the other hand, ePyMT/MSC-Snail1 KO co-xenografts formed tumors with characteristics of adenomas or early carcinomas; thus, epithelial structures were clearly delimited and surrounded by a single layer of stromal cells (Fig 6C).

Tumors were resected and mice maintained alive for one more month until metastases were apparent. All mice orthotopically injected with ePyMT and Snail1 WT MSCs developed lung metastases by this time (Fig 6D-E). Contrarily, mice grafted with epithelial cells and Snail1 KO MSCs did not. Curiously, mice transplanted with only ePyMT generated metastasis in 50% of the cases (Fig 6D). These results indicate that Snail1 expression in tumor fibroblasts is required for tumor invasion and lack of Snail1 in these cells exerts a protective effect.

DISCUSSION

Here, we describe a new role for Snail1 relevant for cancer progression, since it is required for fibroblast activation and for the enhancement of tumor invasion by these cells. Snail1 function has been widely studied in EMT. This factor is required for the onset of the process, the initial repression of *CDH1* and the activation of mesenchymal genes (Thiery et al, 2009). EMT and Snail1 are essential for embryonic development; however, their contribution to epithelial tumor progression is still a matter of discussion. Our analysis of Snail1 expression in human tumors has revealed that, in most neoplasms, it is normally expressed in a limited fraction of cells with fibroblastic morphology and located in the stroma, close to the interface with epithelial tumor cells (Franci et al, 2009; Stanisavljevic et al, 2015). Therefore, we have investigated the function of Snail1 in cancer-associated fibroblasts and its role in epithelial tumor invasion.

Using co-culture models of invasion we demonstrate that mesenchymal cells with characteristics similar to cancer-associated fibroblasts stimulate invasion of epithelial tumor cells. This stimulation is dependent on signals derived from the epithelial cells of the tumor that activate the mesenchymal cell. For most of breast and colon tumor cell lines examined, TGF-β seems to be the cytokine responsible for this activation, although it is possible that other cytokines or growth factors might substitute TGF-β in other tumors. Snail1 expression in fibroblasts is required for a full transcriptional response to TGF-β (Batlle et al., 2013). Snail1 is rapidly induced by this cytokine and is required for the activation of non-canonical targets such as those commonly used as markers for activated fibroblasts or undifferentiated MSCs, two very similar cellular entities. We suggest that the inhibition of the transcriptional activation of TGF-β-target

genes in Snail1 KO cells is likely the consequence of the impaired repression of PTEN by this transcriptional factor (Escrivá et al., 2008; Batlle et al., 2013). Although, as recently reported by us and other groups (Stanisavljevic et al, 2011; Hsu et al, 2014), Snail1 also actively participates in the transcription of mesenchymal genes by binding to their promoters. In any case, it is clear that Snail1 is also required for the stimulation of MSC migration (Fig 1E).

The Snail1-dependent transcriptional program triggered by TGF-β on mesenchymal cells impinges on epithelial invasion on different ways. First, as shown in Fig S4A, it induces the transcriptional activation of several matrix protease genes. Since MSCs precede epithelial cells when invading through the matrix, it is likely that this high expression of proteases helps to degrade the matrix, physically facilitating epithelial cell movement. We have also reported that active MSCs also reorganize the extracellular matrix enabling directional migration of tumor cells (Stanisavljevic et al, 2015).

Besides these indirect actions, MSCs secrete signals like PGE2 that actively facilitate epithelial migration acting as chemo-attractants. Due to the high instability of this molecule, it is likely that this prostaglandin only works at short-range and requires to be produced by cells close to the target cells; thus, mesenchymal cells placed in the tumor-stroma interface. The association of Cox2 expression and PGE_2 levels with cancer malignancy has been previously shown (Menter et al, 2010). Actually, PGE_2 contributes to generate cancer stem cells and stimulate their growth (Li et al, 2012, Kurtova et al, 2015). Although these effects are associated with an EMT (ref??), we did not detect significant morphological alterations in any epithelial cell line after addition of PGE_2; cells maintain E-cadherin expression and invade collectively (Fig S7). Actually, in our model of invasion, MSCs did not cause any significant change in the phenotype

of HT-29 M6 cells that migrate through Matrigel in a collective fashion retaining E-cadherin (Fig 2).

We also demonstrate a role for Snail1 in cancer invasion. First, the effect of tumor-derived CAFs on *in vitro* invasion is associated (for human CAFs) or dependent (for murine CAFs) to Snail1 expression and is partially blocked by TBR or PGE_2 inhibitors. Moreover, Snail1 depletion affects growth and invasion of breast tumors in the PyMT mice model and when comparing tumors of the same size. Those tumors from Snail1 KO mice exhibit a much more differentiated phenotype with fewer areas of invasion and lower lymph node micrometastases. Orthotopic xenograft experiments also showed similar results, since co-injection of epithelial cells with mesenchymal cells depleted for Snail1 originated less advanced and aggressive tumors than when the cells were co-xenografted with Snail1 wild-type cells. Lung metastases were also dependent on Snail1 expression. Curiously, the number of metastases was higher when epithelial cells were injected alone than when cells were injected with Snail1 KO MSCs (Fig 6D), indicating that fibroblasts with loss of Snail1 function protected from tumor invasion. This result is suggesting that Snail1 KO MSCs prevent activation of host fibroblast and inhibit tumor invasion, but it is also possible that the higher secretion of protease inhibitors by these cells also contributes to this inhibition.

We think that these results conclusively demonstrate that Snail1 expression is required for the activation of CAFs and for the effects of these cells on tumor invasion. This agrees with results from our group indicating that Snail1 presence in the stroma associates with a bad prognosis (Francí et al, 2009; Stanisavljevic et al, 2015). However, this conclusion does not mean that Snail1 expression in epithelial cells is not also relevant, since if expressed transiently it also promotes invasiveness in breast

tumor models (Tran et al., 2014). Our results also imply that the overall Snail1 expression in the tumor, considering both epithelial and stromal cells, is not necessarily associated with a loss of E-cadherin since, at least in vitro, expression of Snail1 in the fibroblasts facilitate invasion of tumor cells without a significant EMT. Our results also provide the molecular basis for the association of stromal markers of fibroblast activation with bad prognosis, even in low-grade tumors, since these active fibroblasts are capable to drive collective invasion of tumoral cells, a type of invasion observed in most epithelial neoplasms (ref??).

Finally, due to the positive effect of cancer-associated fibroblasts on epithelial tumor progression and the required expression of Snail1 for their activation, the interference of Snail1 action in fibroblasts emerges as putative target for the action of antineoplastic drugs. Recent reports have indicated that the block of TGF-β receptor might be a promising therapy for colon tumors through the inhibition of tumor-triggered activation of stromal fibroblasts (Calon et al, 2012). Drugs targeting Snail1 present two additional advantages on TGF-β receptor inhibitors: first they would not induce the undesired effects of TGF-β inhibition on tumor progression, since they prevent the antineoplastic action of TGF-b on tumoral cells keeping an intact, not mutated TGF-β signaling response, and second, because Snail1 interference not just prevent the fibroblast activation in response to TGF-β but also to all the extracellular factors we have tested. Therefore, acting as a more general inhibitor of the stimulation of fibroblasts by different tumoral cells.

EXPERIMENTAL PROCEDURES

Reagents

The following reagents were used in this work: TGF-β, PDGF-BB (both from Preprotech), PGE_2 (14010, Cayman Chemical Co), SB505124 (SB; S4696, Sigma), Celecoxib (Pz0008, Sigma), L-161,982 (L-161; SML-0690, Sigma), PF04418949 (PF-044; PZ-0213, Sigma), GM6001 (GM; cc1010, Merck), Crenolanib (S3013, Deltaclon), Cell Tracker (C2925, Life Technologies), DAPI (D9542, Sigma) and Tamoxifen (T5648, Sigma). The antibodies used were: Snail1 (Franci et al, 2006); E-Cadherin (610182, Transduction labs), Tubulin (T9026, Sigma), 15-Hpgd (Ab 967332, Abcam), CK19 (Ab133496, Abcam), and GFP (Ab 6556, Abcam).

Mice

Animals were maintained in a specific pathogen free area and fed ad libitum. All the procedures were approved by the Animal Research Ethical Committee from the Parc de Recerca Biomèdica de Barcelona and by the Generalitat de Catalunya. We have previously described (Batlle et al., 2013) the generation of a murine line with *Snail1*floxed ($Snail1^{Flox}$) and *Snail1* wild-type ($Snail1^{+}$) or *Snail1* deleted ($Snail1^{-}$) alleles and a Cre recombinase-Estrogen Receptor fusion gene under the control of *β-Actin* promoter (β-Actin Cre-ER). These animals were mated with MMTV-PyMT mice (Guy et al, 1992), kindly provided by Dr. Angel Nebreda (IRB, Barcelona). This murine line expresses the Polyoma Virus Middle T antigen under the control of the mouse mammary tumor virus promoter; female mice develop mammary tumors with lung metastases. Depletion of Snail1 in MMTV-PyMT, β-Actin Cre-ER, $Snail1^{Flox/-}$ (or $Snail1^{Flox/+}$ as control) was performed by tamoxifen injection (0.2 mg/g) as described (Batlle et

al., 2013) in eight weeks-old mice. When the experiment lasted more than four weeks, tamoxifen injection was repeated after one month.

Cell Culture

Cells were grown in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 4.5 g/l glucose (Life Technologies), 2 mM glutamine, 56 IU/ml penicillin, 56 mg/l streptomycin, and 10% fetal bovine serum (FBS; GIBCO) and maintained at 37°C in a humid atmosphere containing 5% CO_2. The human colorectal HT-29 M6, HCT-116, SW-620, SW-480, Caco-2 or breast cancer BT-474, MCF-7, MDA-MB231, SK-BR3, T47D cell lines were obtained from ATCC or our institute cell bank. The generation of HT-29 M6 Snail1 cells has been previously reported (Batlle et al, 2000). Mouse embryo fibroblasts (MEFs) and mesenchymal stem cells (MSCs), either Snail1$^{+/-}$ (considered Snail1 wild-type) or Snail1$^{-/-}$ (Snail1 KO) were previously established in our laboratory from the Snail1$^{Flox/-}$ mice (Batlle et al, 2013; Millanes-Romero et al, 2013) by transfection of a plasmid encoding the Cre recombinase or a control vector

Generation and culture of human and murine Cancer-Associated Fibroblasts (CAF) and epithelial PyMT tumor cells (ePyMT).

CAFs: Fresh colon tumor samples were obtained from the Puerta de Hierro University Hospital of Majadahonda. 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. Tissue samples were cut into small pieces of approximately 2–3 mm^3 in size and seeded in FCS medium with

200 u/ml penicillin, 200 μg/ml streptomycin, 100 μg/ml gentamicin, and 2.5 g/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 37ºC in a humid atmosphere containing 5% CO_2. When CAFs reached 70% confluence cells were expanded and cultured in complete medium. Sixteen CAFs populations were analyzed for Snail1 expression; those four with the highest and the lowest Snail1 expression were considered as Snail1-high and Snail1-low human CAFs and used for further assays. Mouse cancer associated fibroblasts were isolated using the same procedure from MMTV-PyMT tumors. Cells were isolated from Snail1$^{Flox/-}$ animals mice in order to obtain MSC Snail1-KO. For this, cells were infected with pMX-Cre retrovirus or the corresponding control and selected with puromycin for 48 hours.

ePyMT: Epithelial cells were isolated from breast tumors from MMTV-PyMT mice model using the mouse tumor dissociation kit (MACS, Miltenyi Biotech) to obtain a single cell suspension. Cells were then incubated with the mouse epithelial cell enrichment kit (STEMCELL Technologies) to eliminate all but epithelial cells; this kit consists in a cocktail of biotinylated monoclonal antibodies against CD45, TER119, CD31 and BP-1 followed by a combination of two mouse lgG monoclonal antibodies against biotin and dextran. After incubation with these antibodies cells are mixed with a suspension of magnetic dextran iron particles and finally with a magnet for 5 min. Epithelial unbound cells were collected and seeded in EpiCult™-B (Mouse) (STEMCELL Technologies} supplemented with EGF (10 ng/mL), FGF (10 ng/mL), Heparin (4 μg/mL) and FBS (2%). Cells were maintained at 37 °C in 5% CO_2 no more than a week.

Invasion Assay

Transwells (3442, Costar) were coated with 50 μL of Matrigel (0.5 μg/μL) (354230, Corning) or Collagen 1 (3 μg/μl) (354249, Corning) and incubated for 2h at 37 °C. For single cells invasion, 0.5-1 x 10^5 epithelial cells or 2 x 10^4 fibroblast were seeded on a Matrigel-coated transwell in DMEM plus FBS (0.1%) and BSA (0.1%) in a final volume of 150 μL. In co-culture experiments RFP-labelled epithelial cells (0.5-1 x 10^5) and GFP-labelled fibroblasts (1/10) were mixed, centrifuged at 300 x g for 5 min, resuspended in 150 μL on the same medium and seeded on Matrigel- or Collagen 1-coated transwells. After 4h at 37 °C, DMEM plus FBS (10%) was added to the lower chamber. At this point, the indicated treatment was added at both chambers except PGE_2, and PDGF-BB, which were added in to the bottom chamber. The invasion was stopped at 48h (epithelial cells) or 24h (fibroblasts); culture medium was kept for other analyses and cells were washed with PBS and fixed with 4% p-formaldehyde for 20 min. Cells at the upper side of the transwell membrane were removed with a cotton swab and the membrane with the invading cells was stained with DAPI and mounted for microscopy analysis. Five random photos (4x) of each membrane were taken to analyze the number of invading cells or the area of invasion in the case of cells that invade in colonies. Only RFP or GFP-labelled cells were analyzed in the co-culture invasion experiments. Alternatively, ePyMT cells were labelled with CellTracker™ Green CMFDA dye (Thermo Fisher; C2925) according to the manufacturer's intructions before mixing with MSCs. Analysis of invading cells was performed with ImageJ software.

Other methods are described in the Supplemental Information

AUTHORS CONTRIBUTIONS

LA-C, RO, AM and RP performed the experiments; LA-C, RO, AM, RP and AGH analyzed the data; MH and CP provided essential tools; FB, CP, JIC, JB and AGH discussed the results; AGH wrote the article.

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.

Figure Legends

Figure 1. **Mesenchymal cell stimulation of HT-29 M6 invasion is dependent on Snail1 and TGF-β.** (**A-C**) HT-29 M6 cells (10^5), labelled with dsRed were seeded alone or with 10^4 MSCs (A-B) or MEFs (C), either wild-type or KO for Snail1 on Matrigel-coated Boyden chambers. When indicated, the TBR inhibitor SB (5 μM) was added to the cell medium. Cells were fixed after 48 hours and samples processed as indicated in Methods. (**D**), TGF-β was determined in the cell medium of Boyden chambers after 48 hours by ELISA (ab119557, Abcam). (**E**) GFP-labelled MSCs were seeded with HT-29 M6 cells or treated with TGF-β (5 ng/ml); when SB was also added. The values were referred to the invasion of MSCs without supplementation. Graphs show the mean ± SEM of at least three independent experiments. * indicates a $p < 0.05$; **, a $p < 0.01$.

Figure 2. **Morphology of the invasion of MSCs/HT-29 M6 co-cultures**. Sections of co-cultured HT-29 M6 and GFP-labelled MSCs were obtained as indicated in Methods, included in paraffin and stained with hematoxilin-eosin (H-E), or analyzed by immunohistochemistry with antibodies against GFP, Snail1, E-cadherin and P-Smad2.

Figure 3. **MSCs enhancement of HT-29 M6 invasion is dependent on PGE_2.** (**A**) The figure shows the secretion of PGE_2 by HT-29 M6 and MSCs (WT or KO for Snail1), cultured on Matrigel-coated Boyden chambers and treated with TGF-β, SB or Celecoxib (1 μM) when indicated. PGE_2 levels were determined by ELISA (RPN222, GE Healthcare Life Science). (**B-E**) Invasion of HT-29 M6 (B-C) or MSCs (D-E) was determined as above either alone or in co-culture with the indicated cells. The following reagents were also supplemented to the medium: Celocoxib, SB (see above), L-161

(10 μM), PF-044 (1 μM), Crenolanib (1 μM), PGE_2 (100 nM) and PDGF-BB (10 ng/ml). Graphs show the mean ± SEM of at least three independent experiments. * indicates a $p < 0.05$; **, a $p < 0.01$

Figure 4. **Stimulation of epithelial cell invasion by CAFs is associated to Snail1 expression**. Murine CAF-1857 and CAF-52149 were obtained from PyMT breast tumors and Snail1 was depleted as indicated in Methods; RNA were obtained and the expression of the indicated genes determined by q-RT-PCR (**A**). The capability of these CAFs population to stimulate invasion of breast dsRed-labelled MCF-7 cells was determined as above with the indicated additions (**B**). (**C**), RNA from four Snail1-high and four Snail1-low CAFs obtained from colon tumors was analyzed for *SNAIL1*, *COX2* and *PTGES2* expression by qRT-PCR. The capability of these clones to stimulate dsRed-labelled HT-29 M6 invasion was determined as above (**D**), as well as the stimulation by HT-29 M6 of their PGE_2 production (**E**). Graphs show the mean ± SEM of three independent experiments. * indicates a $p < 0.05$; **, a $p < 0.01$

Figure 5. **Snail1 depletion retards PyMT breast tumor growth and invasion**. Murine lines were generated holding PyMT, β-Actin-Cre-ER and $Snail1^{Flox/-}$ genes (or $Snail1^{Flox/+}$ as control). Snail1 depletion was carried out in eight weeks-old females and mice were maintained until they breast tumor reach a 1 cm diameter. The survival of Snail1 WT and KO animals is shown in **A** and the tumor burden eight weeks after tamoxifen injection in **B**. Expression of Snail1 protein and hematoxylin-eosin staining of representative sections of both type of tumors in **C** and **D**, respectively. Presence of micrometastases in brachial, axillary, inguinal and sciatic lymph nodes was carried out after staining with an anti CK19 antibody. The percentage of Snail1 WT and KO mice

presenting micrometastases is shown in **E** and a representative staining of one of them in **F**.

Figure 6. **Snail1 expression in MSCs enhances invasion of ePyMT cells *in vivo***. 5 x 10^5 ePyMT and, when indicated, 5 x 10^5 MSCs (Snail1 WT or KO) were injected into the mammary fat pad of a SCID mice. Primary tumors were resected when they reached 1 cm-diameter and their volume (**A**), Snail1 expression (**B**) and histology (**C**) was analyzed. One month after tumor resection mice were euthanized and presence of lung metastases was determined. In **D** the percentage of mice that developed metastases are shown and in **E** a lung colonized by tumor cells. The arrows indicate metastases.

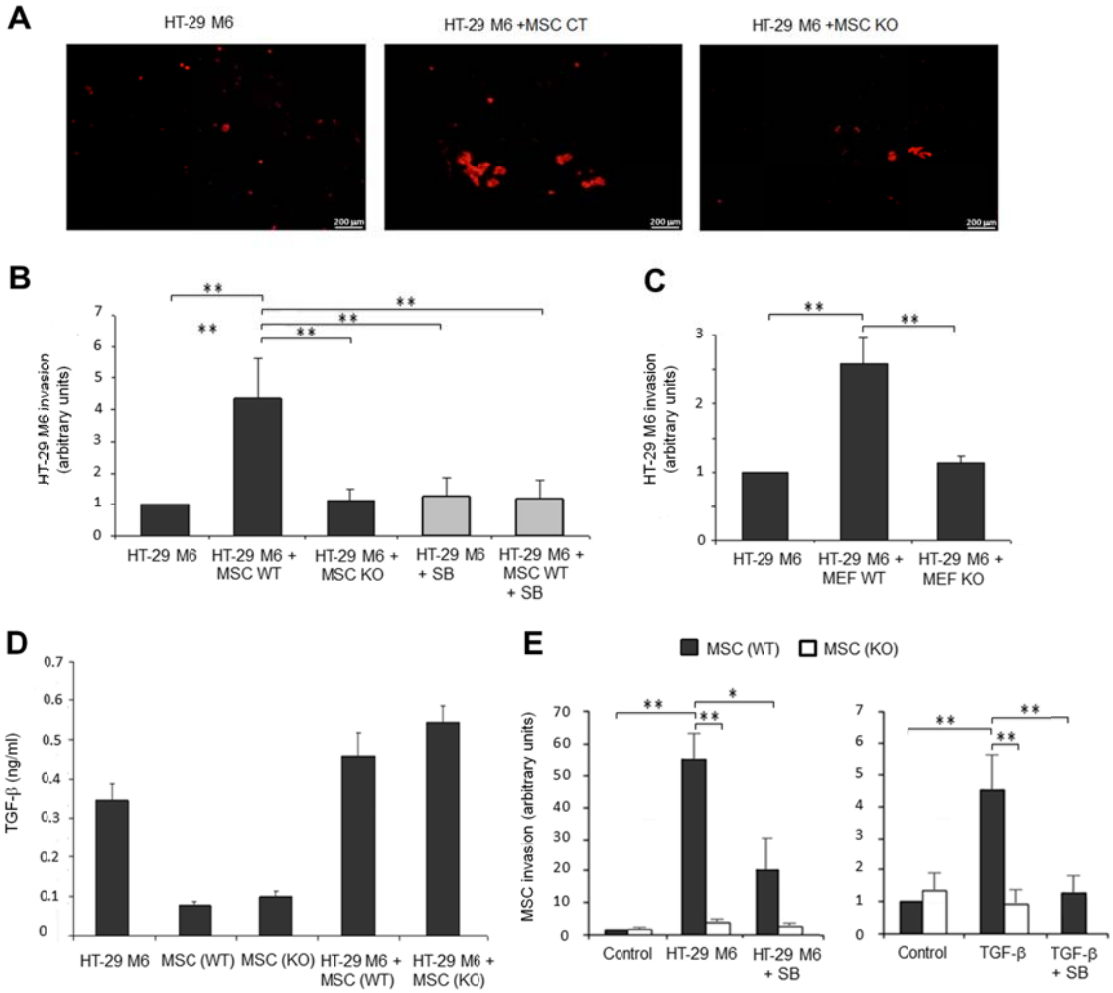
A
HT-29 M6
HT-29 M6 +MSC CT
HT-29 M6 +MSC KO
200 µm
B
HT-29 M6 invasion (arbitrary units)
HT-29 M6
HT-29 M6 + MSC WT
HT-29 M6 + MSC KO
HT-29 M6 + SB
HT-29 M6 + MSC WT + SB
C
HT-29 M6 invasion (arbitrary units)
HT-29 M6
HT-29 M6 + MEF WT
HT-29 M6 + MEF KO
D
TGF-β (ng/ml)
HT-29 M6
MSC (WT)
MSC (KO)
HT-29 M6 + MSC (WT)
HT-29 M6 + MSC (KO)
E
MSC (WT)
MSC (KO)
MSC invasion (arbitrary units)
Control
HT-29 M6
HT-29 M6 + SB
Control
TGF-β
TGF-β + SB

Figure 1

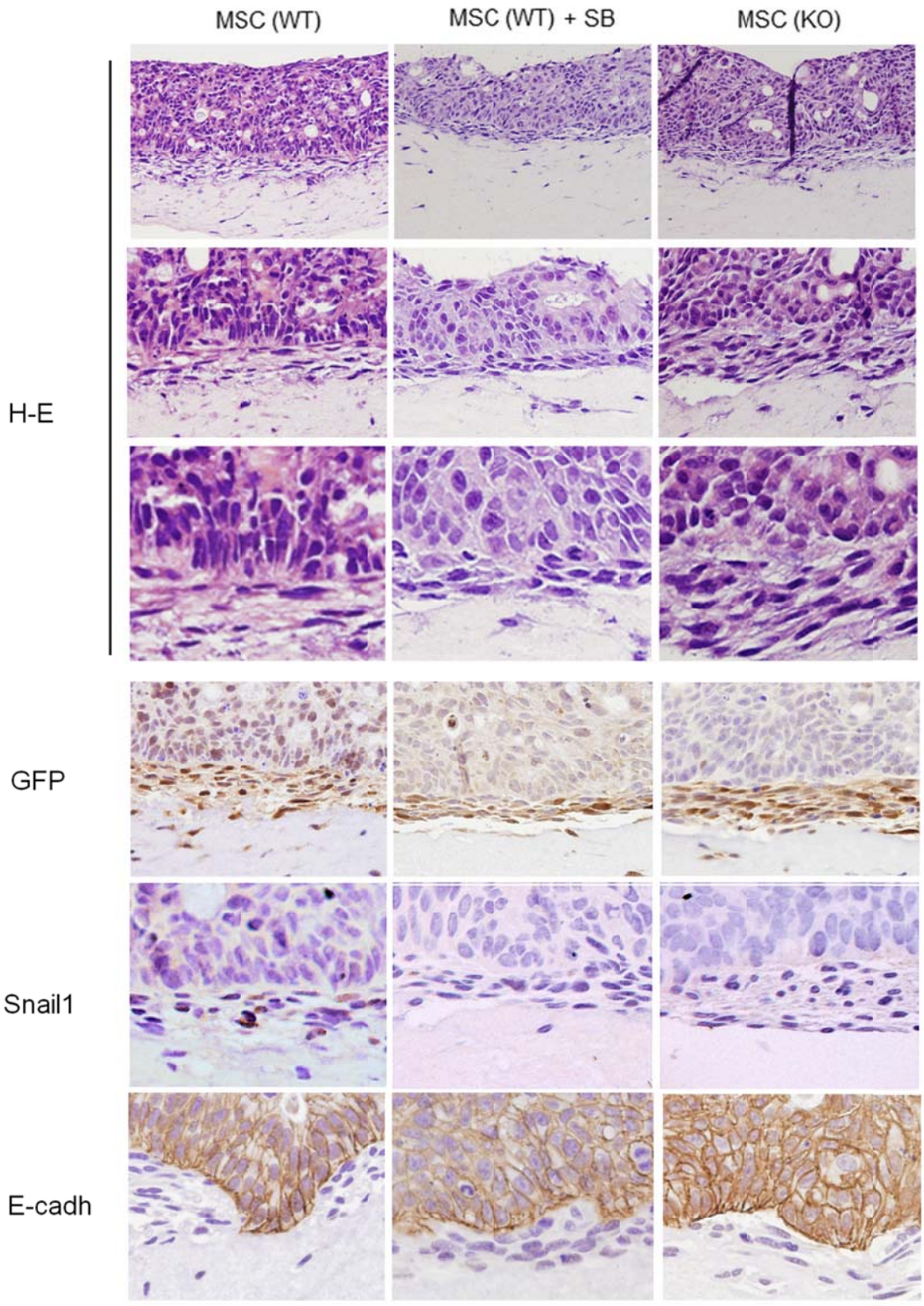

Figure 2

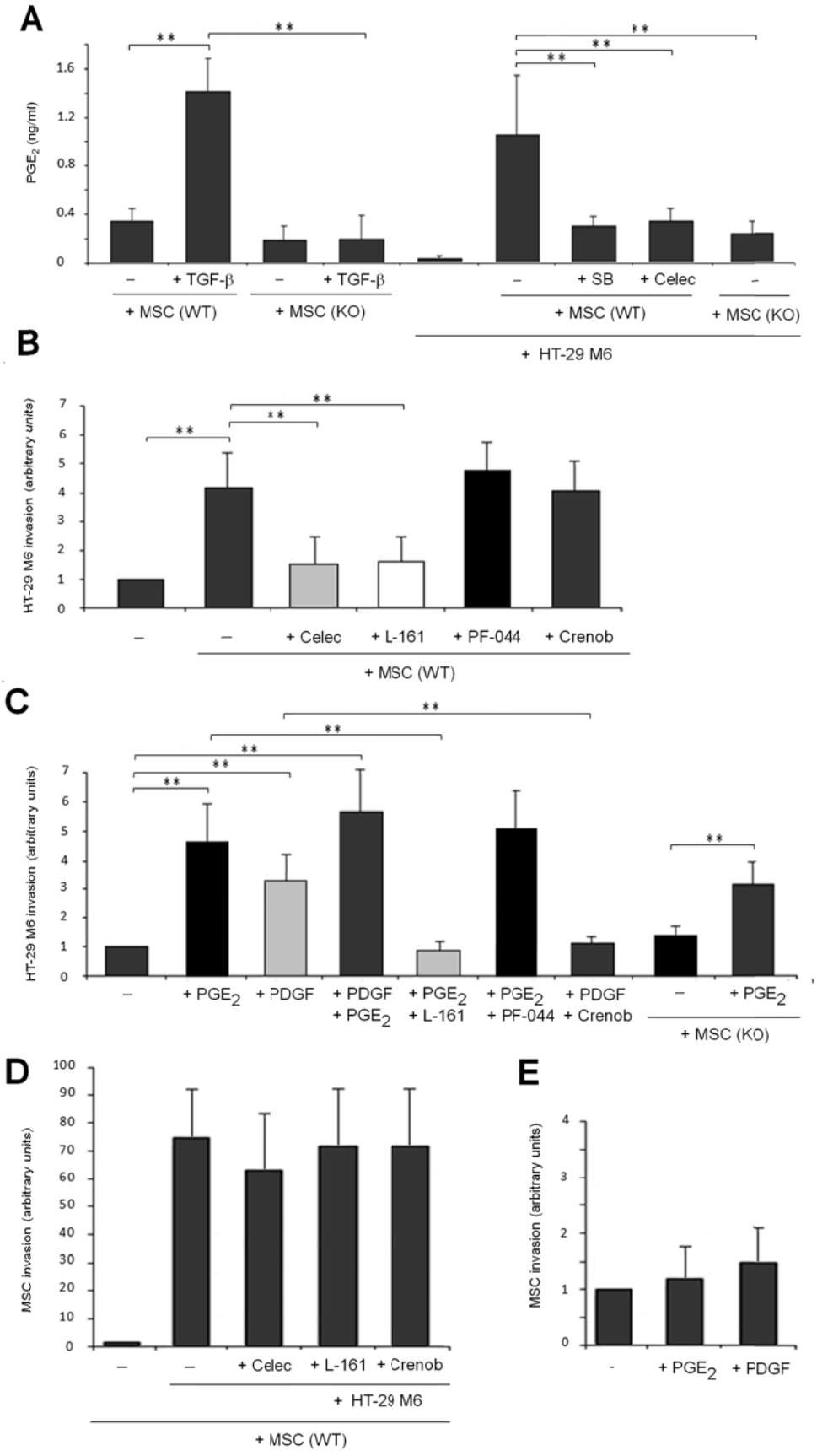
A
PGE2 (ng/ml)
1.6
1.2
0.8
0.4
0
**
**
**
**
**
– + TGF-β – + TGF-β – + SB + Celec –
+ MSC (WT) + MSC (KO) + MSC (WT) + MSC (KO)
+ HT-29 M6
B
HT-29 M6 invasion (arbitrary units)
7
6
5
4
3
2
1
0
**
**
**
– – + Celec + L-161 + PF-044 + Crenob
+ MSC (WT)
C
HT-29 M6 invasion (arbitrary units)
**
**
**
**
**
**
– + PGE2 + PDGF + PDGF + PGE2 + PGE2 + L-161 + PGE2 + PF-044 + PDGF + Crenob – + PGE2
+ MSC (KO)
D
MSC invasion (arbitrary units)
100
90
80
70
60
50
40
30
20
10
0
– – + Celec + L-161 + Crenob
+ HT-29 M6
+ MSC (WT)
E
MSC invasion (arbitrary units)
4
3
2
1
0
- + PGE2 + PDGF

Figure 3

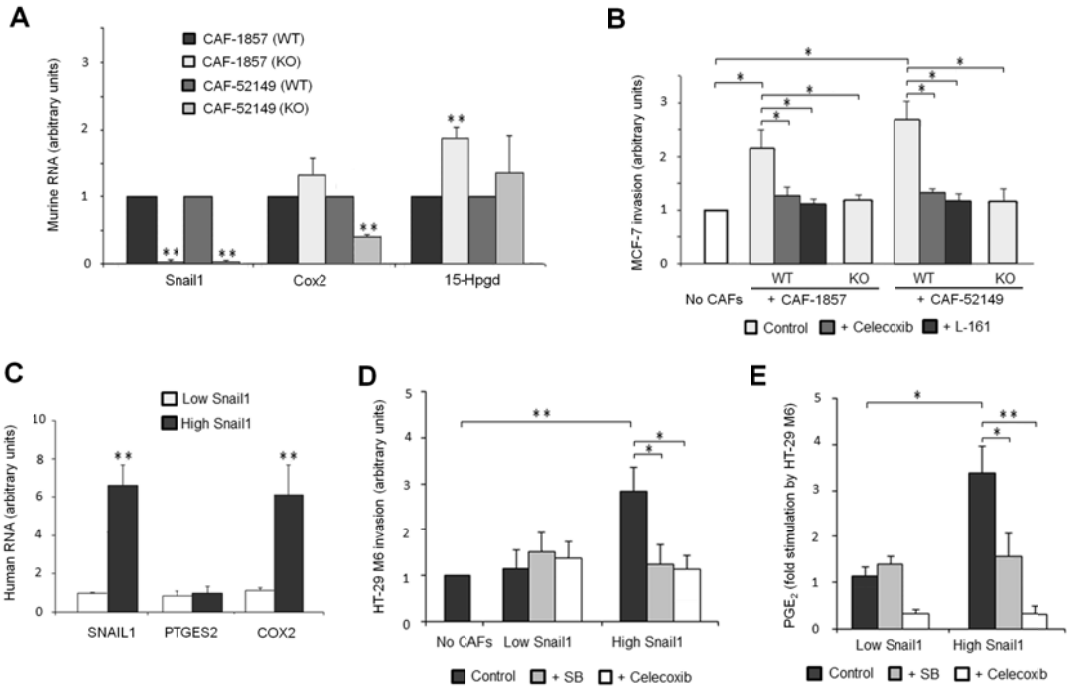
A
CAF-1857 (WT)
CAF-1857 (KO)
CAF-52149 (WT)
CAF-52149 (KO)
Murine RNA (arbitrary units)
Snail1
Cox2
15-Hpgd
B
MCF-7 invasion (arbitrary units)
WT
KO
WT
KO
No CAFs
+ CAF-1857
+ CAF-52149
Control
+ Celecoxib
+ L-161
C
Low Snail1
High Snail1
Human RNA (arbitrary units)
SNAIL1
PTGES2
COX2
D
HT-29 M6 invasion (arbitrary units)
No CAFs
Low Snail1
High Snail1
Control
+ SB
+ Celecoxib
E
PGE2 (fold stimulation by HT-29 M6)
Low Snail1
High Snail1
Control
+ SB
+ Celecoxb

Figure 4

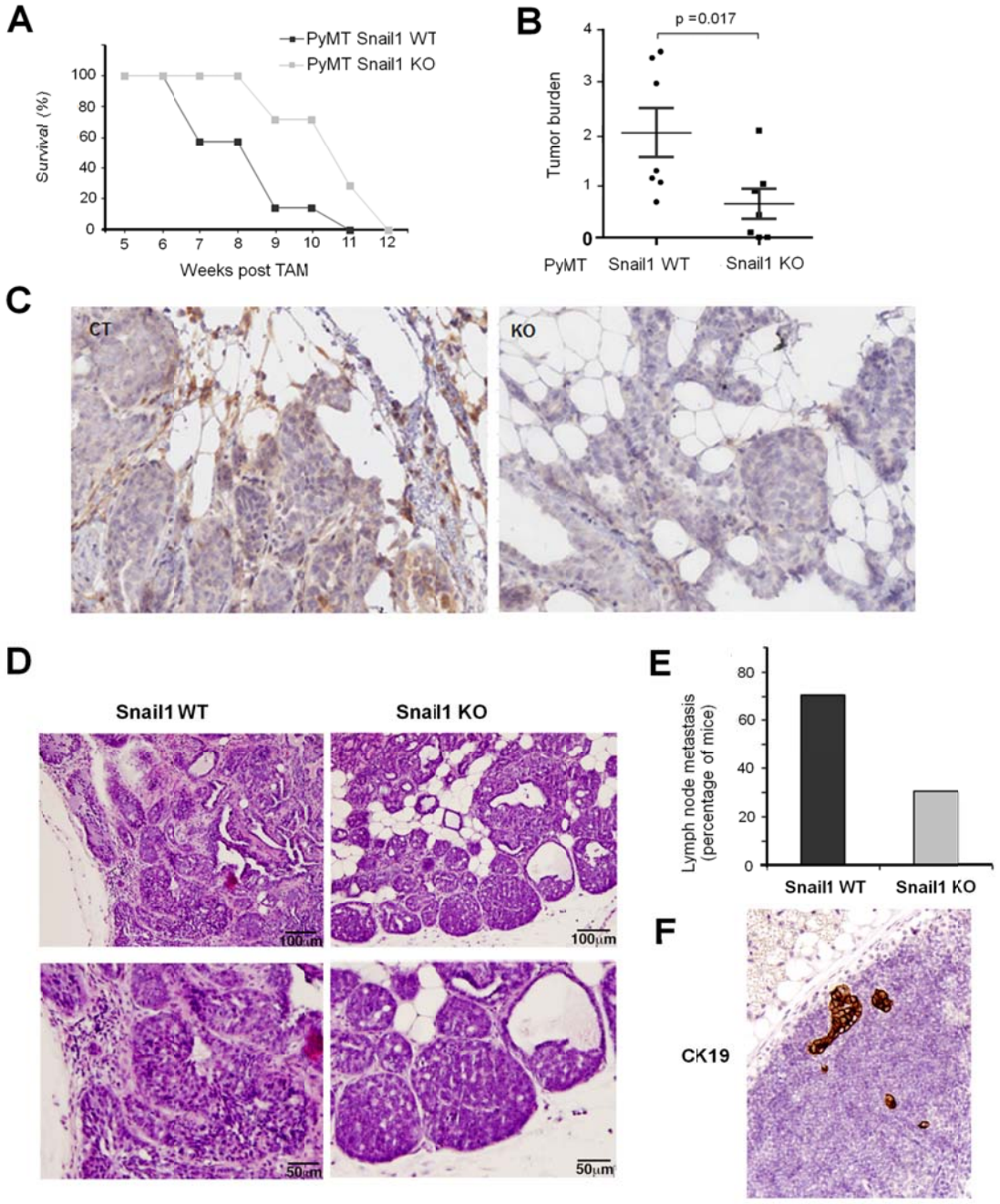
A
PyMT Snail1 WT
PyMT Snail1 KO
Survival (%)
Weeks post TAM
B
p =0.017
Tumor burden
PyMT
Snail1 WT
Snail1 KO
C
CT
KO
D
Snail1 WT
Snail1 KO
100µm
50µm
E
Lymph node metastasis (percentage of mice)
Snail1 WT
Snail1 KO
F
CK19

Figure 5

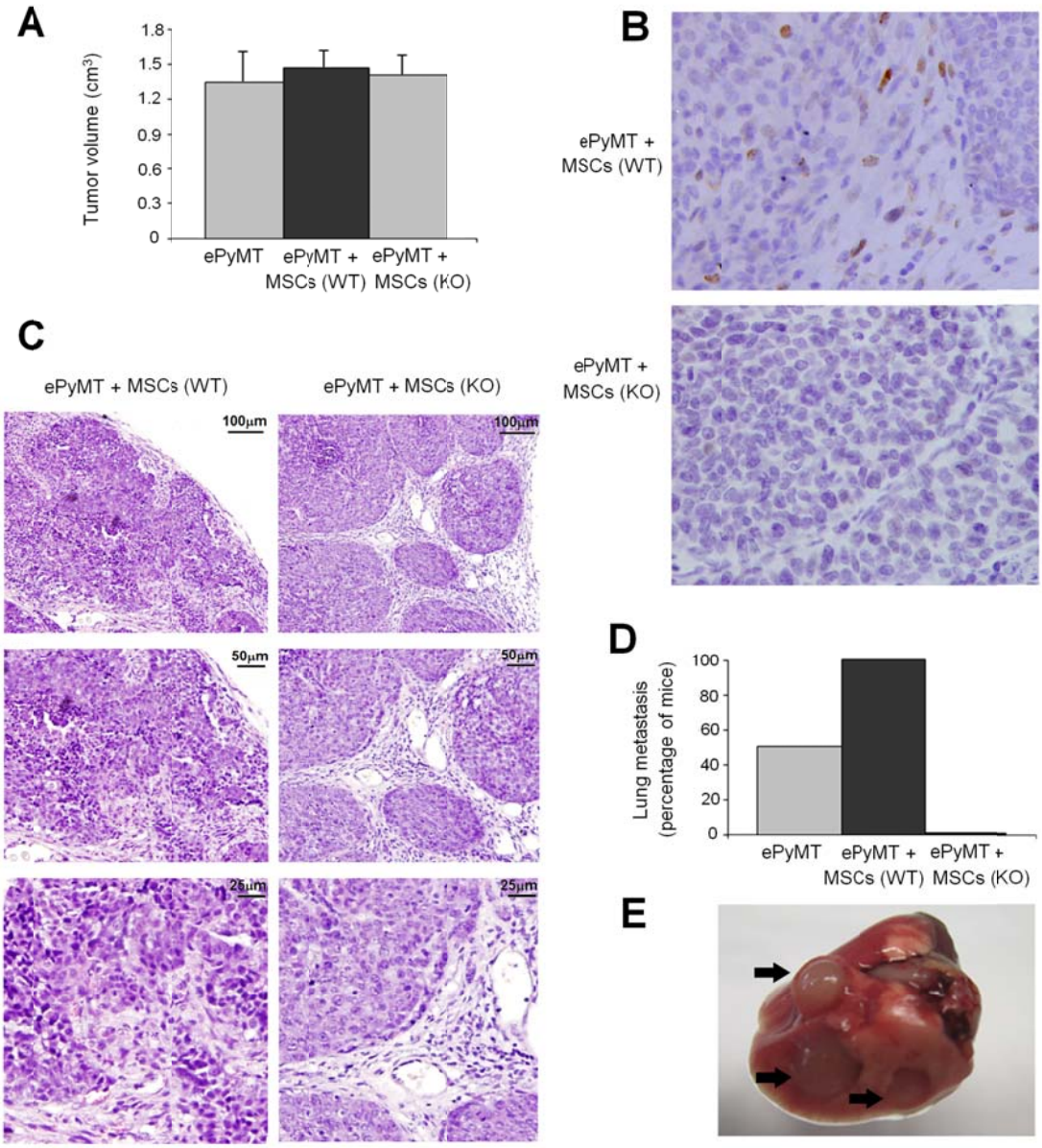
A
Tumor volume (cm3)
1.8
1.5
1.2
0.9
0.6
0.3
0
ePyMT
ePyMT + MSCs (WT)
ePyMT + MSCs (KO)
B
ePyMT + MSCs (WT)
ePyMT + MSCs (KO)
C
ePyMT + MSCs (WT)
ePyMT + MSCs (KO)
100μm
50μm
25μm
D
Lung metastasis (percentage of mice)
100
80
60
40
20
0
ePyMT
ePyMT + MSCs (WT)
ePyMT + MSCs (KO)
E

Figure 6