Snail1-driven plasticity of epithelial and mesenchymal cells sustains cancer malignancy

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Running title: Snail1-Driven Plasticity in Cancer Malignancy

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There are no conflicts of interest to disclose.

Abstract The transcription factor Snail1 induces epithelial-to-mesenchymal transition (EMT) in tumor epithelial cells, a process associated with the emergence of stemness, invasion and cancer malignancy. Here, we review recent reports indicating that Snail1 also regulates mesenchymal plasticity and paracrine signaling and propose that Snail1 orchestrates the generation of cancer stem cells (CSCs) and cancer-associated fibroblasts (CAFs). Our view supports the current models for tumorigenesis that consider stemness and tumor microenvironment as retroactive actors for metastasis formation, revealing Snail1 as a regulator of these metastatic forces. This view offers new perspectives for understanding and targeting metastasis.

1. Introduction

Malignancy in epithelial tumors associates with the formation of metastasis. A simple explanation for how metastasis initiates considers that tumor cells acquire malignant migratory and stem properties by undergoing epithelial-to-mesenchymal transition (EMT). *Snai1* depletion in embryos blocks development at stages that require EMT [1]. Currently, it is broadly accepted that Snail1 is a transcription factor that initiates EMT by directly repressing E-cadherin, a gene essential for maintaining the epithelial phenotype [2]. After undergoing full EMT, the initially static epithelial cells with apicobasal polarity acquire a mesenchymal-like anterior-posterior polarity that allows them to migrate as single units. Similar to other developmental processes involving cell plasticity, EMT has been proposed to be reactivated in tumor cells, allowing these to acquire invasive characteristics (Fig. 1A; ref. 3).

2. Do cancer cells require Snail1 expression and EMT for tumor metastasis?

Although numerous studies have characterized Snail1 expression in a wide variety of cancer cells (reviewed in ref. 4), these results need to be analyzed with caution. Most studies are based on RNA expression, which however does not always reflect protein levels due to the prominent role of post-translational regulation on Snail1 protein stability [5]. Moreover, when extracting RNA from tumors samples, epithelial and stromal components are typically analyzed together, while stromal Snail1 RNA contamination is not taken into account. Finally, most Snail1 protein analyses have been hindered by the poor specificity of available commercial antibodies and by the transient expression of Snail1 during EMT, making it very hard to detect this protein in immunohistochemical analyses of tumor slides. All of these difficulties may explain the discrepant conclusions obtained from these analyses; for instance, in breast tumors, Snail1 expression has been reported to be both significantly [6,7] and not significantly [8,9] associated with patient survival.

On the other hand, a number of oncologists and pathologists remain skeptical about applying the EMT concept to human tumor progression [10]. Their skepticism is supported by the fact that the vast majority of metastatic carcinoma cells show aberrant expression of a few mesenchymal markers yet still retain epithelial features. Moreover, most carcinomas invade by cohesive migration, maintaining an epithelioid cell shape and preserving cell-cell junctions. Other observations suggest that epithelial cells can directly intravasate to tumor-induced neoangiogenic vessels and colonize metastatic tissues starting from the intravascular growth of tumor cells attached to the endothelium, which eventually destroys the vessel wall. Based on these and other observations, some authors argue that EMT may not be a necessary step for metastasis (Fig. 1B; reviewed in 11).

Nevertheless, cell tracking experiments in genetically engineered mouse models (GEMM) support the idea that EMT is relevant in metastasis. A GEMM of pancreatic cancer has identified tagged epithelial cells that exhibit mesenchymal traits in the stroma and bloodstream [12]. These tagged cells colonize the liver, indicating that at least in this metastatic model, epithelial tumor cells can undergo EMT, spread out of the primary tumor and target distant organs [12]. Cell tracking experiments on Snail1-inducible knock-out (KO) mice have also confirmed that Snail1 is required for metastasis. Data from two different GEMMs of breast cancer show that transient expression of Snail1 in tumor cells is necessary for metastatic competence. However, a sustained expression of Snail1 inhibits metastasis [13].

Strong support for the relevance of Snail1 expression and EMT to tumorigenesis comes from recent experiments showing that Snail1 and EMT are required to generate cancer stem cells (CSCs): expression of Snail1 and other EMT-TFs on tumor cells confers stemness [14,15], while Snail1 expression is required to maintain the status of isolated CSCs [16,17]. Properties of CSCs, such as resistance to anoikis and other insults, self-renewal and a multilineage differentiation potential, are required to maintain tumor cells in hostile non-epithelial

microenvironments, to overcome therapeutic insults, and to generate heterogeneous metastatic foci [15]. Thus, although direct evidence of Snail1 protein expression in CSCs within tumors is still missing, Snail1 and EMT have been conceptually associated with the CSC hierarchical model for tumorigenesis.

3. Links between EMT and CSC

Although not always taken into consideration, both stemness and EMT are general concepts that encompass distinct cell states. For instance, the diversity of stem markers expressed in the different cells within a single tissue [18] highlights the distinct populations of stem cells. In these lines, the three main methods of isolating CSC from tumor samples—by flow cytometry using CSC-specific cell surface markers, such as CD133, by Hoechst 33342 dye exclusion that selects the side population phenotype, and by sphere formation in defined medium that maintains undifferentiated CSCs—often give rise to different subpopulations [19]. Accordingly, Brabletz et al. proposed that there are two subpopulations of CSCs that can be distinguished histologically and that have different migratory capacities [20]. They identified the migratory subpopulation, which expresses high nuclear beta-catenin levels and is localized at the invasive tumor front, as the one relevant for metastasis [20].

The terms “partial EMT” and intermediate epithelial-mesenchymal state—like have emerged to define the ambiguous cell status observed in many cancers, with tumoral cells expressing both epithelial and mesenchymal determinants [10,21]. This intermediate state has also been defined by *in silico* approaches. Both stemness and EMT are determined by a binary feedback loop, in which two factors are mutually inhibited. The LIN28/let-7 double inhibition governs stemness, whereas miR-200/ZEB controls EMT. These two modules are coupled, since LIN28 is inhibited by miR-200, and they are both triggered by Snail1, which inhibits let7 [22] and activates ZEB [23]. A mathematical model based on these two central circuits is sufficient to predict the existence of an intermediate state of EMT, which has a high probability of gaining

stemness as compared to the epithelial or mesenchymal state [24]. This model supports the concept that partial EMT is associated with stemness.

Accordingly, it has been proposed that cells acquire stemness along EMT when they reach a suitable status of dedifferentiation; however, they lose this property when they are forced to progress further to a fully committed mesenchymal status. Thus, stemness is maintained by signaling from the niches only within a window between a partially dedifferentiated epithelial status and a partially dedifferentiated mesenchymal status. The relative amounts of structural epithelial and mesenchymal determinants distinguish epithelial stem cells (ESCs) from mesenchymal stem cells (MSCs). ESCs are more static and proliferative, whereas MSCs are migratory [25]. The tumor counterparts of these non-pathological cells are static and migratory CSCs, respectively. An epithelial cell niche, such as that at the bottom of intestinal crypts or at the suprabasal region of breast glands, restricts the CSCs to their static phenotype; however, local micro-environments generated by tumor-stroma at the invasive front can enable migratory CSCs to be generated [26]. Therefore, the EMT gradient model considers different stem and EMT statuses and explains how stemness and EMT are linked (Fig. 2, upper panel).

4. Snail1 promotes epithelial plasticity by controlling the cytoskeleton organization

Molecularly, one of the main elements reorganized by Snail1 during EMT is the cytoskeleton. Cytoskeletal fibers specify cell architecture and are anchored to membrane platforms interacting with extracellular matrix substrates and neighboring cells. Additionally, cytoskeletal and cytoskeletal-associated proteins participate in a wide variety of essential signal transduction pathways that ultimately dictate cell shape and fate. Epithelial cell function is strictly dependent on their apicobasal polarized morphology. This morphology is sustained and regulated by a cortical actomyosin cytoskeletal ring anchored to, and stabilized by, an apical intercellular junctional complex that includes adherens (AJ) and tight junctions (TJ). However, the cytoskeleton is dynamic, thus enabling cell plasticity.

Snail1 expression initiates EMT by acting as a direct transcriptional repressor of cytoskeletal components of both the core proteins of the epithelial junctions, such as E-cadherin from AJ [2] and claudin/occludin from tight junctions TJ [27], and the epithelial intermediate filaments, such as cytokeratin 18 [23]. In addition, Snail1 promotes the activity of RhoA and, through the actions of this GTPase, acts on the contractile cytoskeleton anchored to cell-substrate contacts (within structures termed focal adhesions [FA]), although the molecular details have not been well characterized [28,29]. Cells with highly structured epithelial junctions and cytoskeleton are more resistant to undergoing EMT, since junctions sequester regulatory transcription factors required for Snail1 effects on cell plasticity, such as NF- κ B and β -catenin [30]. The basal lamina of epithelial tissues prevents the first steps of EMT by reinforcing epithelial junctions by activation of the epithelial specific collagen IV receptor, DDR1. However, if the basal lamina is altered, for instance by the deposition of stromal collagen I, EMT is favored by the activation of a DDR2 receptor that promotes Snail1 protein stabilization [31] and by the lack of DDR1 signaling [32].

As mentioned above, initiation of disassembly of the epithelial cytoskeleton allows cells to temporarily acquire a dedifferentiated structure or stem state that can be maintained if they encounter the appropriate stem niche. Static and migratory stem cell states are reversible [26] in a Snail1-dependent manner [33]. Accordingly, cultures of mesenchymal stem cells isolated from human bone marrow express Snail1 [34], and overexpression of ectopic Snail1 on human mammary epithelial produces cells with MSC properties [35]. Thus, within stemness, a sustained Snail1 expression associates with MSCs, while its transitory expression associates with static ESCs (Fig. 2, upper panel).

If EMT advances beyond the stem state, the cytoskeleton adopts a stable organization characteristic of fibroblasts. However, adult fibroblasts do not express Snail1 [36], and their antero-posterior polarity is maintained by transcription factors other than Snail1, such as

Twist, Slug or ZEB1/2 [24,37,38]. Therefore, the final output of EMT is fixed by a Snail1-independent mechanism. Note however that fibroblasts are not the only output of the EMT process (Fig. 2, upper panel). For instance, if TGF β induces Snail1 expression in cultured epithelial renal cells, the cells eventually undergo EMT [39], but if epithelial contacts are disrupted by complementary external inputs—such as wounding or calcium deprivation [40] or in conditions of elevated matrix rigidity [41]—cells undergo epithelial-to-myofibroblast transition (EMyT).

Myofibroblasts (MFs) are a specialized fibroblasts with a cytoskeleton that contains the smooth muscle actin alpha isoform, α SMA [42]. Both a complete disruption of the apical epithelial contacts [43] and extracellular rigidity [41] promote α SMA transcription due to an increase of myocardin-related transcription factor (MRTF). Acquisition of a α SMA-cytoskeleton provides these fibroblasts with strong contractibility, which is very relevant for their capacity to remodel the ECM [42]. MFs in normal or pathological situations, such as in skin wounds [9,36], epithelial cancer lesions [36,44–46], sarcomas [36,47] and fibrotic lesions [48–51], express elevated levels of Snail1. This correlation suggests a role of Snail1 in promoting the α SMA cytoskeleton of MFs (addressed in the next section).

5. Sustained Snail1 expression maintains intra- and extracellular myofibroblastic skeletons

Although both MSCs and MFs express Snail1, MSCs do not express α SMA, so that additional signals to further enhance Snail1 expression, such as induction of the TGF β pathway, are required to promote conversion of MSC to MFs (Fig. 2, upper panel) [9,34,52]. MFs can also transdifferentiate from fibroblasts by sustained activation of Snail1 with TGF β [9,34]. Indeed, MFs are often generated *in vivo* from cytokine-dependent activation of tissue-recruited hematopoietic MSCs or from resident stroma fibroblasts [53–55].

Using cultured fibroblasts, we have recently demonstrated that a sustained TGF β treatment generates MFs that remodel the extracellular matrix in a Snail1-dependent manner [9]. This

remodeling produces abundant fibronectin fibers that are anisotropically aligned and connected with the internal cytoskeleton through mature FA (Fig. 3A). The rigidity of the extracellular matrix (ECM) generated by MFs is dependent on LOX crosslinking of collagen fibers polymerized using fibronectin as a template [56]. Snail1 participates in the transcription of ECM and ECM-modifying molecules, such as fibronectin and LOX, by directly acting as a co-activator of p65-NF κ B [57]. Moreover, Snail1 is required for the activation of RhoA caused by TGF β [9]. In the absence of Snail1, MFs do not form cytoskeletal structures such as α SMA-fibers, mature AJ and FA, or anisotropic extracellular fibronectin fibers. Consequently, the characteristic stiffening of the ECM produced by MFs [56] is not produced in the absence of Snail1 [9].

In MFs, Snail1 levels—and thereby the MF phenotype [58,59]—are auto-sustained by signaling from the tensioned ECM they generate. Thus, mature myofibroblastic FA transmit out-in mechanosensing signals that induce Snail1 expression at the transcriptional level [60–62]. MFs also express DDR2 [63], a collagen I receptor that, when active, prevents Snail1 degradation [31]. On the other hand, the ECM conformational changes produced by MFs can induce the release of latent TGF β forms embedded in the extracellular compartment [64], feeding back to enhance RhoA activity and Snail1 expression. Moreover, MFs activate autocrine feed-back loops to produce more TGF β [65] in a Snail1-dependent fashion (Fig. 3B). Therefore, Snail1 and RhoA activities are maintained by feedback signaling from the extracellular compartment generated by MFs. This cell-specific property perpetuates the myofibroblastic phenotype.

6. Relevance of fibroblasts expressing Snail1 in cancer

A population of MFs associated to tumors, termed cancer-associated fibroblasts (CAFs), participates in establishing a dynamic tumor-microenvironment crosstalk required for the onset of metastasis. CAFs are recruited and activated by factors derived from neoplastic cells, mimicking non-pathological processes such as wound healing, where these recruiting factors

are secreted from injured epithelial cells and macrophages [66]. Subsequently, by secreting soluble factors and remodeling the architecture of the ECM, CAFs favor stemness, migration of tumor cells and eventually metastasis [66].

Several studies have focused on Snail1 expression in CAFs by analyzing its correlation with cancer progression. At early stages (I and II) of colon cancers, Snail1 expression in the tumor stroma correlates with poor survival [36]; similarly, in early infiltrating breast cancer at diagnosis, the presence of few Snail1-expressing CAFs in the tumor stroma associates with lymph node involvement and poor outcome [9]. Snail1 expression in endothelial cells and tumor stromal myofibroblasts also predicts poor survival in pharyngeal squamous cell carcinomas [67].

In infiltrating breast cancers, the presence of even a low number of Snail1-expressing CAFs in the tumor stroma correlates with the appearance of desmoplastic areas with anisotropic fibers. Accordingly, in cultured CAFs lines, Snail1 levels correlates with the levels of fibronectin, α SMA, and N-cadherin and with the degree of tridimensional organization of ECM fibers. Co-cultures with low percentages of CAFs are sufficient to educate non-activated naive fibroblasts to produce an organized ECM resembling that observed *in vivo*. Rigid and organized matrices promote not only anisotropic tumor cell movement and increased invasiveness but also EMT and stemness [9,68–70]. A similar control of the extracellular architecture by Snail1-expressing fibroblasts is also observed in wound healing. Skin wound closure is delayed in Snail1 KO-induced mice that present an unusual granulocyte tissue including fibroblasts with low α SMA expression and a disorganized fiber structure compared to control animals, which have Snail1-positive fibroblasts in this tissue [9].

Additionally, CAFs produce a characteristic secretome that is also Snail1-dependent. Thus, the levels of Snail1 expressed by different CAF lines established from colon cancer patients associate with a cytokine profile and with a capacity to induce migration of colon tumor cell in

a paracrine manner [46]. In a further study, the protein-secreted profile of TNF α stimulated cancer cells was shown to be Snail1-dependent [71]. TNF α promotes the CBP-dependent acetylation of Snail1, which regulates the transcription and secretion of two chemokines, CCL2 and CCL5, involved in recruitment of tumor-associated macrophages (TAMs). The presence of acetylated Snail1 and TAMs in head and neck cancers associates with low patient survival [71]. Therefore, Snail1 activity controls the pro-tumorigenic paracrine signaling generated by both activated tumor cells and CAFs.

We propose that although only few tumor cells and CAFs express detectable levels of Snail1, this can be sufficient to induce metastasis through a mechanism involving mutual cooperation. The malignant properties described to be a consequence of Snail1 expression in tumor cells (migration, invasiveness and stemness) are indirectly induced by the activity of CAFs that express Snail1. Indeed, rigid ECMs and exocrine signaling, generated by Snail1-expressing CAFs, favor Snail1 expression in tumor cells [72]. It is plausible that a Snail1-dependent bi-directional paracrine signaling between epithelial tumor cells and mesenchymal host cells activates the Snail1-dependent plastic processes affecting these cells. These reciprocal effects on plasticity, which generates cooperative phenotypes requiring Snail1, may also occur during liver repair. In this organ, tissue injuries switch Snail1 on in hepatocytes, promoting their growth and producing an inflammatory response [49]; these inflammatory signals induce Snail1 in mesenchymal hepatic stellate cells, potentiating their contractility and their production of additional signals required for hepatocyte regeneration [73].

7. Conclusion

By mutually coordinating the onset of plastic processes in tumor and stromal cells, Snail1 governs the tumor fate. In tumor cells, Snail1 expression promotes cytoskeleton changes required for the partial activation of the EMT program, providing them with two essential metastatic properties: stemness and motility. In non-tumoral stroma cells, such as fibroblasts

or tumor-recruited MSCs, tumor-derived cytokines induce Snail1 expression and promote the acquisition of myofibroblastic traits. Thus, Snail1-expressing CAFs can sustain a physically and chemically pro-metastatic micro-environment. Through this circuit between tumor and stromal cells, Snail1 contributes to tumor progression in a more potent fashion to what was initially proposed. However, it is possible that in certain tumor types, activation of only a part of these Snail1-dependent events may be sufficient to promote tumor progression. For instance, in pancreatic cancer, Snail1-induced EMT and stemness may be enough for the tumor cells to spread to the blood stream, whereas in most cancers, the activity of fibroblasts expressing Snail1 is also required. For the future, we have the immediate goals of accurately detecting Snail1 in tumors, elucidating its role in cell plasticity, and characterizing the paracrine signaling observed in the different type of tumors, all of which will provide new clues for the mechanisms leading to invasion and dissemination of epithelial neoplasms. Elucidating these details will be critical for determining, and then exploiting, the potential of Snail1 as a pharmacological target.

Acknowledgements

We thank Dr. J Stanisavljević for images (Fig. 2A) and conceptual input provided when she was a PhD student in JB's lab, and Drs. V.M. Díaz and S. Peiró for comments. Work of Drs. Baulida and García de Herreros' labs is supported by the Instituto Carlos III (PI12/00257 and RD012/0036/0005, part of the Plan Nacional I+D+I and cofounded by the ISCIII-Subdirección General de Evaluación and Fondo Europeo de Desarrollo Regional-FEDER), the Fundación Científica de la Asociación Española contra el Cáncer and the Ministerio de Economía y Competitividad (SAF2013-4889-C2-1R).

References

1. Carver EA, Jiang R, Lan Y, Oram KF, Gridley T. The mouse snail gene encodes a key regulator of the epithelial-mesenchymal transition. *Mol Cell Biol.* 2001;21:8184–8.
2. Batlle E, Sancho E, Francí C, Domínguez D, Monfar M, Baulida J, et al. The transcription factor snail is a repressor of E-cadherin gene expression in epithelial tumour cells. *Nat Cell Biol.* 2000;2:84–9.
3. Hay ED. An overview of epithelio-mesenchymal transformation. *Acta Anat (Basel).* 1995;154:8–20.
4. Kaufhold S, Bonavida B. Central role of Snail1 in the regulation of EMT and resistance in cancer: a target for therapeutic intervention. *J Exp Clin Cancer Res.* 2014;33:62.
5. Díaz VM, Viñas-Castells R, García de Herreros A. Regulation of the protein stability of EMT transcription factors. *Cell Adh Migr.* 2014;8:418–28.
6. Elloul S, Elstrand MB, Nesland JM, Tropé CG, Kvalheim G, Goldberg I, et al. Snail, Slug, and Smad-interacting protein 1 as novel parameters of disease aggressiveness in metastatic ovarian and breast carcinoma. *Cancer.* 2005;103:1631–43.
7. Blanco MJ, Moreno-Bueno G, Sarrio D, Locascio A, Cano A, Palacios J, et al. Correlation of Snail expression with histological grade and lymph node status in breast carcinomas. *Oncogene.* 2002;21:3241–6.
8. Nassar A, Sookhan N, Santisteban M, Bryant SC, Boughey JC, Giorgadze T, et al. Diagnostic utility of snail in metaplastic breast carcinoma. *Diagn Pathol.* 2010;5:76.
9. Stanisavljevic J, Loubat-Casanovas J, Herrera M, Luque T, Pena R, Lluch A, et al. Snail1-expressing fibroblasts in the tumor microenvironment display mechanical properties that support metastasis. *Cancer Res.* 2014;75:284–95.
10. Savagner P. The epithelial-mesenchymal transition (EMT) phenomenon. *Ann Oncol.* 2010;21 Suppl 7:vii89–92.
11. Chui MH. Insights into cancer metastasis from a clinicopathologic perspective: Epithelial-Mesenchymal Transition is not a necessary step. *Int J Cancer.* 2013;132:1487–95.
12. Rhim AD, Mirek ET, Aiello NM, Maitra A, Bailey JM, McAllister F, et al. EMT and dissemination precede pancreatic tumor formation. *Cell.* 2012;148:349–61.
13. Tran HD, Luitel K, Kim M, Zhang K, Longmore GD, Tran DD. Transient SNAIL1 Expression Is Necessary for Metastatic Competence in Breast Cancer. *Cancer Res.* 2014;74:6330–40.
14. Mani SA, Guo W, Liao M-J, Eaton EN, Ayyanan A, Zhou AY, et al. The epithelial-mesenchymal transition generates cells with properties of stem cells. *Cell.* 2008;133:704–15.

15. Hollier BG, Evans K, Mani S a. The epithelial-to-mesenchymal transition and cancer stem cells: a coalition against cancer therapies. *J Mammary Gland Biol Neoplasia*. 2009;14:29–43.
16. Gomez-Casal R, Bhattacharya C, Ganesh N, Bailey L, Basse P, Gibson M, et al. Non-small cell lung cancer cells survived ionizing radiation treatment display cancer stem cell and epithelial-mesenchymal transition phenotypes. *Mol Cancer*. 2013;12:94.
17. Wang H, Zhang G, Zhang H, Zhang F, Zhou B, Ning F, et al. Acquisition of epithelial-mesenchymal transition phenotype and cancer stem cell-like properties in cisplatin-resistant lung cancer cells through AKT/ β -catenin/Snail signaling pathway. *Eur J Pharmacol*. 2014;723:156–66.
18. Medema JP. Cancer stem cells: the challenges ahead. *Nat Cell Biol*. Nature Publishing Group, a division of Macmillan Publishers Limited. All Rights Reserved.; 2013;15:338–44.
19. Akunuru S, James Zhai Q, Zheng Y. Non-small cell lung cancer stem/progenitor cells are enriched in multiple distinct phenotypic subpopulations and exhibit plasticity. *Cell Death Dis*. 2012;3:e352.
20. Brabletz T, Jung A, Spaderna S, Hlubek F, Kirchner T. Opinion: migrating cancer stem cells - an integrated concept of malignant tumour progression. *Nat Rev Cancer*. 2005;5:744–9.
21. Klymkowsky MW, Savagner P. Epithelial-mesenchymal transition: a cancer researcher's conceptual friend and foe. *Am J Pathol*. 2009;174:1588–93.
22. Unternaehrer JJ, Zhao R, Kim K, Cesana M, Powers JT, Ratanasirintrawoot S, et al. The Epithelial-Mesenchymal Transition Factor SNAIL Paradoxically Enhances Reprogramming. *Stem Cell Reports*. 2014;3:691–8.
23. Guaita S, Puig I, Franci C, Garrido M, Dominguez D, Batlle E, et al. Snail induction of epithelial to mesenchymal transition in tumor cells is accompanied by MUC1 repression and ZEB1 expression. *J Biol Chem*. 2002;277:39209–16.
24. Jolly MK, Huang B, Lu M, Mani SA, Levine H, Ben-Jacob E. Towards elucidating the connection between epithelial-mesenchymal transitions and stemness. *J R Soc Interface*. 2014;11:20140962.
25. Ombrato L, Malanchi I. The EMT universe: space between cancer cell dissemination and metastasis initiation. *Crit Rev Oncog*. 2014;19:349–61.
26. Liu S, Cong Y, Wang D, Sun Y, Deng L, Liu Y, et al. Breast cancer stem cells transition between epithelial and mesenchymal states reflective of their normal counterparts. *Stem cell reports*. 2014;2:78–91.
27. Ikenouchi J, Matsuda M, Furuse M, Tsukita S. Regulation of tight junctions during the epithelium-mesenchyme transition: direct repression of the gene expression of claudins/occludin by Snail. *J Cell Sci*. 2003;116:1959–67.

28. Bhowmick NA, Ghiassi M, Bakin A, Aakre M, Lundquist CA, Engel ME, et al. Transforming growth factor-beta1 mediates epithelial to mesenchymal transdifferentiation through a RhoA-dependent mechanism. *Mol Biol Cell*. 2001;12:27–36.
29. McGrail DJ, Mezencev R, Kieu QMN, McDonald JF, Dawson MR. SNAIL-induced epithelial-to-mesenchymal transition produces concerted biophysical changes from altered cytoskeletal gene expression. *FASEB J*. 2014;
30. García de Herreros A, Baulida J. Cooperation, amplification, and feed-back in epithelial-mesenchymal transition. *Biochim Biophys Acta*. Elsevier B.V.; 2012;1825:223–8.
31. Zhang K, Corsa CA, Ponik SM, Prior JL, Piwnica-Worms D, Eliceiri KW, et al. The collagen receptor discoidin domain receptor 2 stabilizes SNAIL1 to facilitate breast cancer metastasis. *Nat Cell Biol*. 2013;15:677–87.
32. Koenig A, Mueller C, Hasel C, Adler G, Menke A. Collagen type I induces disruption of E-cadherin-mediated cell-cell contacts and promotes proliferation of pancreatic carcinoma cells. *Cancer Res*. 2006;66:4662–71.
33. Jiang H, Lin X, Liu Y, Gong W, Ma X, Yu Y, et al. Transformation of epithelial ovarian cancer stemlike cells into mesenchymal lineage via EMT results in cellular heterogeneity and supports tumor engraftment. *Mol Med*. 2012;18:1197–208.
34. Batlle R, Alba-Castellón L, Loubat-Casanovas J, Armenteros E, Francí C, Stanisavljevic J, et al. Snail1 controls TGF- β responsiveness and differentiation of mesenchymal stem cells. *Oncogene*. 2013;32:3381–9.
35. Battula VL, Evans KW, Hollier BG, Shi Y, Marini FC, Ayyanan A, et al. Epithelial-mesenchymal transition-derived cells exhibit multilineage differentiation potential similar to mesenchymal stem cells. *Stem Cells*. 2010;28:1435–45.
36. Francí C, Takkunen M, Dave N, Alameda F, Gómez S, Rodríguez R, et al. Expression of Snail protein in tumor-stroma interface. *Oncogene*. 2006;25:5134–44.
37. Tran DD, Corsa CAS, Biswas H, Aft RL, Longmore GD. Temporal and spatial cooperation of Snail1 and Twist1 during epithelial-mesenchymal transition predicts for human breast cancer recurrence. *Mol Cancer Res*. 2011;9:1644–57.
38. Casas E, Kim J, Bendesky A, Ohno-Machado L, Wolfe CJ, Yang J. Snail2 is an essential mediator of Twist1-induced epithelial mesenchymal transition and metastasis. *Cancer Res*. 2011;71:245–54.
39. Moustakas A, Heldin C-H. Induction of epithelial-mesenchymal transition by transforming growth factor β . *Semin Cancer Biol*. 2012;22:446–54.
40. Masszi A, Fan L, Rosivall L, McCulloch CA, Rotstein OD, Mucsi I, et al. Integrity of cell-cell contacts is a critical regulator of TGF-beta 1-induced epithelial-to-myofibroblast transition: role for beta-catenin. *Am J Pathol*. 2004;165:1955–67.

41. O'Connor JW, Riley PN, Nalluri SM, Ashar PK, Gomez EW. Matrix rigidity mediates TGF β 1-induced epithelial-myofibroblast transition by controlling cytoskeletal organization and MRTF-A localization. *J Cell Physiol*. 2014;
42. Hinz B. The myofibroblast: paradigm for a mechanically active cell. *J Biomech*. Elsevier; 2010;43:146–55.
43. Charbonney E, Speight P, Masszi A, Nakano H, Kapus A. β -catenin and Smad3 regulate the activity and stability of myocardin-related transcription factor during epithelial-myofibroblast transition. *Mol Biol Cell*. 2011;22:4472–85.
44. Francí C, Gallén M, Alameda F, Baró T, Iglesias M, Virtanen I, et al. Snail1 protein in the stroma as a new putative prognosis marker for colon tumours. Callaerts P, editor. *PLoS One*. Public Library of Science; 2009;4:e5595.
45. Herrera M, Islam ABMMK, Herrera A, Martín P, García V, Silva J, et al. Functional heterogeneity of cancer-associated fibroblasts from human colon tumors shows specific prognostic gene expression signature. *Clin Cancer Res*. 2013;19:5914–26.
46. Herrera A, Herrera M, Alba-Castellón L, Silva J, García V, Loubat-Casanovas J, et al. Protumorigenic effects of Snail-expression fibroblasts on colon cancer cells. *Int J Cancer*. 2014;134:2984–90.
47. Alba-Castellón L, Batlle R, Francí C, Fernández-Aceñero MJ, Mazzolini R, Peña R, et al. Snail1 expression is required for sarcomagenesis. *Neoplasia*. 2014;16:413–21.
48. Nakamura M, Tokura Y. Expression of Snail1 in the fibrotic dermis of postmenopausal frontal fibrosing alopecia: possible involvement of an epithelial-mesenchymal transition and a review of the Japanese patients. *Br J Dermatol*. 2010;162:1152–4.
49. Rowe RG, Lin Y, Shimizu-Hirota R, Hanada S, Neilson EG, Greenson JK, et al. Hepatocyte-derived Snail1 propagates liver fibrosis progression. *Mol Cell Biol*. 2011;31:2392–403.
50. Balli D, Ustiyani V, Zhang Y, Wang I-C, Masino AJ, Ren X, et al. Foxm1 transcription factor is required for lung fibrosis and epithelial-to-mesenchymal transition. *EMBO J*. 2013;32:231–44.
51. Liu Y, Du J, Zhang J, Weng M, Li X, Pu D, et al. Snail1 is involved in de novo cardiac fibrosis after myocardial infarction in mice. *Acta Biochim Biophys Sin (Shanghai)*. 2012;44:902–10.
52. Kim W, Barron DA, San Martin R, Chan KS, Tran LL, Yang F, et al. RUNX1 is essential for mesenchymal stem cell proliferation and myofibroblast differentiation. *Proc Natl Acad Sci U S A*. 2014;111:16389–94.
53. Lekkerkerker AN, Aarbiou J, van Es T, Janssen RAJ. Cellular players in lung fibrosis. *Curr Pharm Des*. 2012;18:4093–102.
54. Xu J, Liu X, Koyama Y, Wang P, Lan T, Kim I-G, et al. The types of hepatic myofibroblasts contributing to liver fibrosis of different etiologies. *Front Pharmacol*. 2014;5:167.

55. Mack M, Yanagita M. Origin of myofibroblasts and cellular events triggering fibrosis. *Kidney Int.* 2014;
56. Singh P, Carraher C, Schwarzbauer JE. Assembly of fibronectin extracellular matrix. *Annu Rev Cell Dev Biol.* 2010;26:397–419.
57. Stanisavljevic J, Porta-de-la-Riva M, Batlle R, de Herreros AG, Baulida J. The p65 subunit of NF- κ B and PARP1 assist Snail1 in activating fibronectin transcription. *J Cell Sci.* 2011;124:4161–71.
58. Amatangelo MD, Bassi DE, Klein-Szanto AJP, Cukierman E. Stroma-derived three-dimensional matrices are necessary and sufficient to promote desmoplastic differentiation of normal fibroblasts. *Am J Pathol.* 2005;167:475–88.
59. Hinz B. Tissue stiffness, latent TGF- β 1 activation, and mechanical signal transduction: implications for the pathogenesis and treatment of fibrosis. *Curr Rheumatol Rep.* 2009;11:120–6.
60. Barberà MJ, Puig I, Domínguez D, Julien-Grille S, Guaita-Esteruelas S, Peiró S, et al. Regulation of Snail transcription during epithelial to mesenchymal transition of tumor cells. *Oncogene.* 2004;23:7345–54.
61. McPhee TR, McDonald PC, Oloumi A, Dedhar S. Integrin-linked kinase regulates E-cadherin expression through PARP-1. *Dev Dyn.* 2008;237:2737–47.
62. Goodwin JM, Svensson RU, Lou HJ, Winslow MM, Turk BE, Shaw RJ. An AMPK-independent signaling pathway downstream of the LKB1 tumor suppressor controls Snail1 and metastatic potential. *Mol Cell.* 2014;55:436–50.
63. Márquez J, Olaso E. Role of discoidin domain receptor 2 in wound healing. *Histol Histopathol.* 2014;29:1355–64.
64. Klingberg F, Chow ML, Koehler A, Boo S, Buscemi L, Quinn TM, et al. Prestress in the extracellular matrix sensitizes latent TGF- β 1 for activation. *J Cell Biol.* 2014;207:283–97.
65. Kojima Y, Acar A, Eaton EN, Mellody KT, Scheel C, Ben-Porath I, et al. Autocrine TGF- β and stromal cell-derived factor-1 (SDF-1) signaling drives the evolution of tumor-promoting mammary stromal myofibroblasts. *Proc Natl Acad Sci U S A.* 2010;107:20009–14.
66. Öhlund D, Elyada E, Tuveson D. Fibroblast heterogeneity in the cancer wound. *J Exp Med.* Rockefeller University Press; 2014;211:1503–23.
67. Jouppila-Mättö A, Tuhkanen H, Soini Y, Pukkila M, Närkiö-Mäkelä M, Sironen R, et al. Transcription factor snail1 expression and poor survival in pharyngeal squamous cell carcinoma. *Histol Histopathol.* 2011;26:443–9.
68. Lee H-O, Mullins SR, Franco-Barraza J, Valianou M, Cukierman E, Cheng JD. FAP-overexpressing fibroblasts produce an extracellular matrix that enhances invasive velocity and directionality of pancreatic cancer cells. *BMC Cancer.* 2011. page 245.

69. Karagiannis GS, Poutahidis T, Erdman SE, Kirsch R, Riddell RH, Diamandis EP. Cancer-associated fibroblasts drive the progression of metastasis through both paracrine and mechanical pressure on cancer tissue. *Mol Cancer Res.* 2012;10:1403–18.
70. Cukierman E, Bassi DE. Physico-mechanical aspects of extracellular matrix influences on tumorigenic behaviors. *Semin Cancer Biol.* 2010;20:139–45.
71. Hsu DS-S, Wang H-J, Tai S-K, Chou C-H, Hsieh C-H, Chiu P-H, et al. Acetylation of snail modulates the cytokinome of cancer cells to enhance the recruitment of macrophages. *Cancer Cell.* 2014;26:534–48.
72. Leight JL, Wozniak MA, Chen S, Lynch ML, Chen CS. Matrix rigidity regulates a switch between TGF- β 1-induced apoptosis and epithelial-mesenchymal transition. *Mol Biol Cell.* 2012;23:781–91.
73. Scarpa M, Grillo AR, Brun P, Macchi V, Stefani A, Signori S, et al. Snail1 transcription factor is a critical mediator of hepatic stellate cell activation following hepatic injury. *Am J Physiol Gastrointest Liver Physiol.* 2011;300:G316–26.

Fig. 1. Simple EMT dependent and independent metastasis models. A) EMT-dependent model. Static tumor epithelial cells acquire migratory and invasive properties by EMT. Single cells generated by EMT escape from primary tumors, intravasate, and extravasate at distant organs. A subsequent reversion of EMT, a mesenchymal-to-epithelial transition (MET), is required to generate an epithelial metastasis. B) in the EMT-independent model, neoangiogenic vessels that infiltrate tumors are susceptible to be intravasated by clusters of epithelial tumor cells that, once attached to capillary endothelia of distant organs, can grow and eventually extravasate. Metastasis originates from these epithelial cells. All of these processes require signaling from activated host cells, which are not depicted here. Green cells: cells undergoing EMT or acquiring stemness properties that express or may have expressed Snail1 at the onset of their activation.

Fig. 2. Snail1 expression along cancer-associated cell plasticity. **Upper panel:** EMT/MET is a cell plasticity process with intermediate phenotypes associated with stemness. In a non-pathological epithelial tissue, most of the cells are fixed in a differentiated status by the epithelial cortical cytoskeleton. However, signaling of cytokines such as TGF β , which induces Snail1 expression and partially disassembles the epithelial structure, allow cells within stem niches maintain an intermediate EMT status with stem characteristics. If this signal is discontinued, cells progress backwards towards differentiated epithelial lineages. When EMT proceeds further by the action of external signals that maintain Snail1 expression, cells acquire a mesenchymal structure that can eventually differentiate into fibroblasts or myofibroblasts. In contrast to fibroblasts, which require factors other than Snail1 to set their mesenchymal structure, myofibroblasts require continuous Snail1 expression to generate a highly contractile α SMA-cytoskeleton. MF-remodeled extracellular microenvironment promotes feedback signaling that sustains Snail1 expression and myofibroblastic phenotype. **Middle panel:** In tumor cells, the onset of EMT within stem cell niches produces stationary CSCs that can feed heterogeneous epithelial tumors. Tumor stroma-induced local microenvironments rich in cytokines and fibrotic molecules, such as collagen I, produce the appropriate conditions to stimulate Snail1 and generate migratory CSCs. At distant organs, local microenvironments partially reverse this EMT to produce stationary CSCs, which favors growth of metastatic foci resembling the primary tumor. **Lower panel:** Generation of Snail1-expressing CAFs with myofibroblastic traits from resident or tumor-recruited MSCs is dependent on factors secreted by tumor and tumor-activated cells. Once activated, CAFs reorganize the ECM and establish self-sufficient signals necessary for maintaining their contractile skeleton and phenotype. Micro-environmental changes generated by CAFs favor cancer cell migration and metastasis.

A

B

Fig. 3. Snail1 expression and extracellular architecture are mutually sustained in MFs. A) tridimensional reconstruction of confocal immunofluorescence images showing cellular and matrix alignment of TGF β -activated fibroblasts. Fibronectin staining is shown in red, α SMA, in green, and nuclei (DAPI), in blue (J. Stanisavljevic and J.B., unpublished observations). B) feedback loops sustaining Snail1 expression in MFs. External stimuli that induce MFs, such as TGF β , initiate the expression of Snail1 and activate RhoA. Neither TGF β nor Snail1 alone can fully activate RhoA, and their downstream effectors are responsible for formation of active α SMA stress fibers, maturation of FA, ordered fibronectin-fibrillogenesis, and collagen assembly and crosslinking. Snail1 also mediates the transcription of ECM molecules that favors the formation of a rich fibrillar microenvironment typical of fibrotic and desmoplastic tissues. Feedback mechanisms sustaining Snail1 levels and myofibroblastic phenotype are activated by the remodeled ECM, including mechano-transduction through FA, activation of collagen DDR2 receptors, and release of latent TGF β through conformational changes associated to increased stiffness. Fibronectin fibers are shown in pink, collagen fibers, in violet, SMA stress fibers, in green, and mature FAs, in red.