Snail family regulation and epithelial mesenchymal transitions in breast cancer progression.

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1. Summary and goal of the review

Since its initial description (1), the interconversion between epithelial and mesenchymal cells (designed as epithelial-mesenchymal or mesenchymal-epithelial transition, EMT or MET, respectively) has received special attention since it provides epithelial cells with migratory features. Different studies using cell lines have identified cytokines, intercellular signaling elements and transcriptional factors capable of regulating this process. Particularly, the identification of Snail family members as key effectors of EMT has opened new ways for the study of this cellular process. In this article we discuss the molecular pathways that control EMT, showing a very tight and interdependent regulation. We also analyze the contribution of EMT and Snail genes in the process of tumorigenesis using the mammary gland as cellular model.

2. EMT: general characteristics, stages of EMT found *in vivo* and *in vitro*. Partial EMT.

EMT consists of a rapid and often reversible change in the cell phenotype. Epithelial cells lose their cell-cell adhesion structures including adherens junctions and desmosomes, modulate their polarity and rearrange their cytoskeleton: intermediate filaments typically revert to vimentin from keratins. Cells become isolated, motile, and resistant to apoptosis. EMT was originally defined in the context of developmental stages, including heart morphogenesis, mesoderm and neural crest formation. In the last twenty years EMT has been studied in detail *in vitro* allowing the characterization of numerous pathways, involving growth/differentiation factors such as EGF, FGF, TGF-β and the Wnt pathway, as well as transcriptional factors such as Snail genes, Twist, Zeb genes and E47.

Lately, the EMT concept has been broadened to include a similar transition, called partial EMT. This partial EMT takes place physiologically during wound healing and mammary tubulogenesis (2), and leads to an intermediate phenotype, retaining some characteristics of epithelium but also showing features of mesenchymal cells. During these two processes, cells spread and migrate actively but maintain some cell-cell cohesiveness allowing the so-called cohort migration, in contrast to cells ongoing a complete EMT. Cell-cell adhesion structures such as adherens junctions and desmosomes are reorganized with a sparse distribution. Cytoskeleton composition changes, but cells maintain cytokeratin expression. Similar pathways including

growth/differentiation factors, as well as transcriptional factors such as Slug are involved in cutaneous wound healing (2).

During mammary morphogenesis, tubules grow through a process involving a terminal end bud driven by cap cells. These cells express specific cadherins (P-cadherin) and lead the growing tubule through proliferation and active migration. Signalling pathways including Wnt and other EMT-related pathways are overactive within this pluripotential population giving rise to myoepithelial and luminal cells. One remarkable feature of this morphogenetic program is the link between several cellular processes. Partial EMT is one aspect of the program, however, it can not been dissociated from proliferation, differentiation and apoptosis control. The link between EMT and stemness has been recently explored in several publications (3, 4), and emphasizes the relevance of this intermediate "metastable" phenotype (5), for generating plasticity, but also in transcriptional reprogramming. It is important to remark that the final result of EMT *in vivo* is not usually a fibroblast, but a partially dedifferentiated cell, probably better fit than an epithelial cell to migrate or to respond to an aggression.

EMT or partial EMT processes are also observed during tumor progression and emergence of metastasis. However, due to the absence of direct clinical evidence for EMT, some pathologists are not convinced about the relevance of this transition in cancer progression. We agree that a distinction must be made between EMT *sensu stricto* and the EMT phenotype observed in carcinoma. Since the poor differentiation typically expressed by tumoral cells might result from a faulty differentiation process as well as EMT, it appears more appropriate to use the term of "EMT-like" to describe the phenotype observed in the tumors. Using this terminology, we have distinguished four EMT stages ranging from the full EMT found in carcinosarcoma to the prevalent partial-EMT phenotype expressed in most ductal invasive carcinomas (6). This grading system does not take into account metastatic cells that separate from the initial tumor.

Many genes and pathways, including Snail genes, have been implicated in EMT in tumor cells (see below). Typically, these pathways are also active in other processes including cell proliferation, apoptosis and differentiation during early developmental stages, tissue morphogenesis and wound healing. Their specific role during human tumor progression is not yet well understood. We discuss how we envision this pathway for the control of the expression of the epithelial gene E-cadherin.

3. EMT and E-cadherin down-regulation: role of transcriptional repressors.

The gene encoding the adherens junction protein E-cadherin is considered to be the paradigmatic epithelial gene. Experiments with transgenic animal models have determined that loss of this gene is associated to higher invasion and metastasis (7). Forced expression of E-cadherin in cultured tumor cells deficient for this protein induces a more epithelial phenotype decreasing migration (8-10); moreover, ectopic expression of this protein also prevents the transcription of mesenchymal genes (11, 12). Therefore, E-cadherin loss is normally considered the main hallmark of EMT.

In general, E-cadherin is not expressed in mesenchymal cells as a consequence of the action of transcriptional repressors (13, 14). Besides this transcriptional control, E-cadherin protein stability is also finely tuned: functional E-cadherin, which is present in mature adherens junctions, is highly stable, whereas when not associated to the cytoskeleton the protein is much more labile (15, 16). Therefore, not all the epithelial cells will be equally sensitive to the action of stimuli triggering EMT, since cells where E-cadherin is present in very stable adherens junctions will not be affected by these stimuli. In these cells, repression of E-cadherin gene expression will not cause, or will very slowly cause, a down-regulation of E-cadherin protein. Thus, only epithelial cells where the adhesive function has been altered ("primed" epithelial cells) will be suitable to down-regulate E-cadherin and undergo an EMT. Priming may be a consequence of the action of factors promoting adherens junction disassembly, such as the transcriptional factor FoxC1 (17), the Ras oncogene (18), or the adhesion molecule L1 (19), likely acting through the stimulation of tyrosine kinases.

E-cadherin gene (CDH1) expression is modulated by several transcriptional repressors, which are typically expressed in mesenchymal cells. These factors bind to specific sequences in the CDH1 promoter that contain a central core 5'-CACCTG-3' and are denominated E-boxes. In the tumor cell lines lacking E-cadherin, mutation of these elements stimulates CDH1 gene expression by interfering the binding of specific transcriptional repressors (13, 14). In 2000, two independent reports demonstrated that Snail factor is capable of binding these elements and repressing E-cadherin, consequently inducing an EMT (20, 21). Since then, a plethora of other transcriptional factors have been characterized as CDH1 repressors and EMT inducers although only six are capable of directly interacting with the CDH1 promoter. Besides KLF8, which binds to GT boxes (22), the other five repressors associate to the E-boxes; these are the two members of the Snail family (Snail/Snail1 and Slug/Snail2) (20, 21, 23), the two Zeb proteins (zeb1 and 2) (24, 25) and the bHLH protein E47 (26).

The bHLH family displays a common protein structural domain consisting of two parallel amphipatic α-helices linked by a loop that is required for dimerization. bHLH transcription factors bind to DNA as homo- or heterodimers through a consensus E-box site (5'-CANNTG-3') (27). Among these factors, only E47 (also known as TCF3) has been found to be capable of binding and repressing CDH1 (26). However, the relevance of this factor in EMT has been very little studied, as well as its induction by stimuli triggering this conversion.

The ZEB family of transcription factors consists of two members: ZEB1 (also known as TCF8 and δEF1) and ZEB2 (ZFXH1B and SIP1) (28). Their protein structure is characterized by the presence of two zinc finger domains and a homeodomain. The zinc finger domains are located at both ends of the protein and contain from three to four zinc fingers of the C_2H_2 and C_3H type. The homeodomain is located in the middle part of the protein. The members of this family interact with the DNA through the simultaneous binding of the two zinc-finger domains to E-boxes (28). Both proteins are strong repressors of E-cadherin. Although they are not as potent as Snail in the induction of EMT or in the repression of E-cadherin in *in vitro* assays (37), their silencing, especially that of Zeb1, has a higher impact on E-cadherin expression than Snail (25, 28, 29). E-cadherin transcriptional repression has been associated to the existence in Zeb1 and Zeb2 of a binding element for CtBP, an ubiquitous co-repressor (28). Accordingly, knock-out of CtBP1 or functional sequestering of this protein by Pnn/DRS or E1A relieves E-cadherin repression in tumor cells (30-33). Moreover, inhibition of Zeb1 and 2 expression by the miR200 family restores E-cadherin protein expression, supporting the role of these repressors in the control of CDH1 transcription (38-42).

4. Snail proteins as essential regulators of EMT

Snail transcriptional repressors, and particularly Snail, are the most widely studied effectors of EMT and CDH1 expression. Snail family members form part of the Snail superfamily of transcription factors, composed by the SNAIL and the SCRATCH family (34). The three vertebrate members belonging to the SNAIL family have been called SNAIL (SNAIL1), SLUG (SNAIL2) and the less characterized SMUC (SNAIL3). All the family members encode transcription factors of the zinc-finger type. They share a similar organization with a highly conserved C-terminal domain, which contains from

four to six C_2H_2 type zinc fingers (in a variable number among the different homologues) and bind to the E-box 5'-CACCTG-3' (or in the inverse form, 5'-CAGGTG-3') (34). When compared, Snail binds to these sequences with a higher affinity than Slug and is a more potent inhibitor of CDH1 and other target genes (35). Among these, Snail prevents the expression of epithelium-specific genes such as PTEN, Muc1, Claudin, and Occludin as well as some nuclear factor receptors (Vitamin D receptor, HNF-1α) (36).

Not all the Snail targets are related to EMT. As mentioned above, besides having been associated to tumor invasion, EMT and Snail have been related to other cancer hallmarks such as the gain of unlimited replication potential, a greater resistance to apoptosis and even with the evasion of immunosurveillance. For instance, cell lines that over-express Snail show lower apoptosis when exposed to ionizing radiation, genotoxic drugs or pro-apoptotic cytokines (37-39). Repression of proapoptotic genes such as PTEN, p53, Bid or DFF40 have been associated to this resistance (38, 39). Moreover, Snail also enables breast cells to become tumor-initiating cells (3, 4) and promotes immunosupression in melanoma cells (40). The precise targets of Snail involved in these effects are currently unknown.

The N-terminal half of Snail is responsible for the fine control of this transcriptional factor activity. This part of the protein (in human or murine Snail comprising amino acids 1-150, see figure 1) is much more divergent and holds several relevant sequences. First, a SNAG subdomain, placed at the N-terminus of all vertebrate Snail proteins (34), is required for the binding of co-repressors: the chromatin remodelling factors histone deacetylases (HDAC) 1 and 2 (41) and the Polycomb group of proteins 2 (PRC2) (42). The interaction of these proteins with Snail is not direct but mediated by other proteins, Sin3a and Ajuba (41, 43), respectively. In Drosophila, repression does not seem to require the SNAG domain but a binding site for CtBP (44), which is not present in the mammalian genes. These results suggest that although the repressor activity of the SNAIL family members has been evolutionary conserved, they use different mechanisms depending on either SNAG or CtBP binding elements.

Snail can also bind other proteins. For instance it has been recently shown that Snail co-immunoprecipitates with a Smad2/3 complex and that this complex is present on the CDH1 promoter when transcription of this gene is repressed (45). The CDH1 promoter contains a Smad-binding element (SBE) close to one of the E-boxes; a similar

proximity of E-boxes and SBE has also observed for other Snail target genes such as CAR (45) and PTEN (SP and AGH, unpublished observations).

Snail not only represses epithelial genes but also stimulates mesenchymal gene transcription. It has been proposed that Snail stimulatory effects are dependent on the repression of E-cadherin and the release of transcriptional factors retained by this protein; accordingly E-cadherin over-expression prevents Snail induction of mesenchymal genes (12). However, stimulation of gene transcription can not be exclusively explained by E-cadherin inhibition since genetic interference of CDH1 transcription does not promote the activation of mesenchymal genes to the same extent as Snail expression. Moreover, Snail effects on mesenchymal genes are detected even in cells defective for expression of E-cadherin (12). It has also been reported that Snail interacts with β-catenin in the nucleus (46) promoting transcriptional activation of Wnt target genes, suggesting that Snail, at least in certain conditions, might work as a direct activator.

The central part of the Snail proteins is involved in the regulation of protein stability and localization. Different phosphorylation motifs have been allocated in this domain. For instance, phosphorylation of Snail on Ser 104 and 107 by GSK3β has been proposed to facilitate Snail nuclear exit, unmasking a nuclear export sequence placed between amino acids 132 and 143. Once in the cytosol, further phosphorylation on Ser 96 and 100 by the same protein kinase (47) induces Snail binding to β-TrCP1 ubiquitin ligase, leading to its ubiquitination and degradation (48). This phosphorylation is counteracted by the action of the small C-terminal domain phosphatase (SCP), which stabilizes Snail in the nucleus (49). Snail can also undergo phosphorylation in other residues that positively control Snail action. For instance, phosphorylation of Ser 11 and 92 by protein kinase A and CK2, respectively, stimulate Snail repression of E-cadherin and interaction with Sin3A co-repressor (50). Moreover, the C-terminus of Snail can be phosphorylated by PAK1 (51) resulting in increased protein retention in the nucleus.

It has also been shown that the interaction of Snail with Lysil oxidase-like 2 (LOXL2) also affects its repressive function through the oxidation of Lys 98 and 137 by this enzyme (52). These authors propose that LOXL2 catalyzes the oxidative deamination of the two lysines, leading to the formation of a covalent cross-link and inducing a conformational change that would mask GSK3β phosphorylation motifs and prevent further degradation.

Snail is an unstable protein with a half-life from 20 to 45 minutes. Besides β-TrCP1, that requires the previous phosphorylation of the protein, we have recently described another E3 ubiquitin ligase, FBXL14, that interacts with Snail in cell lines and promotes its ubiquitination and proteosome degradation (53). Fbxl14 interacts with Snail through amino acids 120-151, also present in the central domain of the protein but, in contrast to β-TrCP1, its binding is not dependent on the previous phosphorylation. Curiously, both ubiquitin ligases act through the modification of Lys 138 and 146 (53).

Compared to Snail, the biochemical characteristics of the rest of the members of the Snail family, Slug and Smuc, have been less studied. As mentioned, Slug is not as potent as Snail repressing E-cadherin and binds to E-boxes in this promoter with lower affinity (35). Repression also requires the interaction of histone deacetylases with the N-terminal regulatory domain (54). Curiously these authors reported that a small sequence in this domain activates transcription in GAL4 fusion proteins. Slug lacks most of the phosphorylation sites in the central part of the molecule. Maybe for this reason ectopic Slug is exclusively detected in the nucleus with a specific dotted staining (54); however these data need to be confirmed when reliable antibodies become available. Although it is not phosphorylated and therefore it does not bind β-TrCP1 ubiquitin ligase, the stability of Slug protein is also tightly controlled. In Xenopus, Slug protein half-life is controlled by the Fbxl14 ortholog Partner of Paired (Ppa) that interacts with a hydrophobic sequence in the central part of the molecule (55). Moreover, Slug protein has also been shown to be a target of the Mdm2 ubiquitin ligase (56). Binding of this enzyme to Slug takes place through amino acids 27-66 and is dependent on p53 that also associates to Slug in a neighbor sequence (amino acid 21-27) (56).

Smuc also shares a similar structure, with a C-terminal DNA binding domain and an N-terminal regulatory domain (57). The three proteins of this family present an almost identical SNAG subdomain placed in the very N-terminal end. However, so far it has not been reported that proteins interacting to the SNAG sequence in Snail, for instance, Ajuba or Sin3a, also associate to Slug or Smuc. The similarity in the DNA-binding domain is high and most of the Snail target promoters can also be bound by Slug *in vitro*, although with a lower affinity. Conversely, few cases have been reported for a Slug target that can not be bound by Snail; only the antiapoptotic protein Puma seems to gather these conditions (39, 58). The lack of *in vivo* Slug association to some Snail target promoters could be explained by the lower affinity shown by Slug and might

explain the less complete (or more reversible) EMT detected after expression of this transcriptional factor in epithelial cells (59, 60).

5. Control of Snail protein expression: cooperation and feedback.

Up-regulated SNAIL gene expression has been detected in most experimental conditions in which cells in culture are forced to adopt a mesenchymal phenotype; for instance, after long treatments with cytokines such as TGF-β or Interleukin 6 or by over-stimulation of receptor tyrosine kinases (36). However, we still have limited information about the factors controlling Snail promoters. Contrary to codifying sequences, promoter homology tends to be low. Comparative analysis of the Snail and Slug promoters shows the presence of conserved and functional response elements, such as AP1 and AP4 sites, SMAD-binding elements, LEF1 binding sites and E-boxes (36). Only in some cases the relevance of these elements is well established: for example, hepatocyte growth factor (HGF) targets the early growth response 1 gene (Egr1) protein to the SNAIL promoter and activates its expression through the activation of mitogen activated protein kinase 1 (MAPK1) (61). The cellular factors involved in the response to TGF-β have also been investigated. Activation of SNAIL transcription is dependent on the physical interaction of the high mobility group A2 (HMGA2) protein with Smads, which increases the Smad binding to the SNAIL promoter (62). Moreover, an enhancer located 3' of the SNAIL open reading frame is also relevant for the expression of the gene (63).

Snail binds and directly represses its own promoter (64). The Snail-binding sequence, an E-box, is conserved in mouse, rat, macaque, bovine, fruitfly and zebrafish. This repression of its own synthesis creates a feedback loop, a regulation particularly relevant in cellular pathways involved in embryonic development (65). The self-inhibitory effects of Snail are not only limited to the association to its own promoter since Snail can also bind to the promoter and inhibit the synthesis of Egr1, a Snail transcriptional activator mentioned above. These reactions create a robust inhibitory feedback control of Snail expression that can provide cells with the capability of buffering, meaning to stabilize Snail levels in spite of small perturbations in its transcription. As far as this is concerned, many reported effectors of Snail expression are factors, such as ERK2 or PI3K, stimulated in conditions that do not lead to an EMT. Therefore, self-inhibition avoids that transient increases in ERK2 in epithelial cells induce a sustained activation of Snail protein and the subsequent phenotypic changes.

Surprisingly, Slug is able to activate its own promoter during neural crest development by interacting with an E-box (66). Such observation contradicts the paradigm of Snail family of transcription factors behaving as transcriptional repressors upon direct binding to E-boxes and remains, to date, uncertain. However, the fact that Snail can interact with the transcriptional activator β-catenin and increase the expression of Wnt targets suggests that this factor might be also recruited to promoters of activated genes, although not necessarily through the binding to E-boxes. In addition to these data, several reports suggest that Snail can also activate its own synthesis. For example, some of the genes induced after Snail ectopic expression correspond to proteins capable of increasing the expression of Snail gene. This is the case for SPARC; it has been reported to be activated by Snail (67) and also to be an inductor of Snail synthesis and EMT (68). It is likely that this complex regulation is a consequence of the mutual regulation of Snail and NF-κB, a transcriptional factor with a key role in EMT (69). It has been demonstrated both that NF-κB is an inductor of Snail expression (70-72) and that Snail increases NF-κB transcriptional activity and its binding to target genes (12). At this moment it is not totally clear how Snail up-regulates NF-κB transcriptional activity. Moreover, we have detected that ectopic Snail increases its own synthesis in a cell-dependent manner. These specific effects are associated to the E-cadherin levels since E-cadherin over-expression promotes the predominance of Snail self-inhibition. The negative effects of E-cadherin on the activity of NF-κB have been described by several labs (12, 73). Moreover, ectopic expression of Snail in keratinocytes has been shown to activate ERK2, whereas reintroduction of E-cadherin in these cells restores the activity of this kinase to basal levels (74).

Finally, a positive feedback loop can also been inferred considering the capability of Snail to block the synthesis of its inhibitors. For instance, Snail binds to the Estrogen Receptor α (ER-α) promoter and down-regulates the expression of this gene (75). At the same time, activation of ER-α is necessary for the expression of MTA-3, an inhibitor of Snail transcription (76). All these data indicate the existence of a positive feedback regulation of Snail expression that may help to integrate the different signals required for the induction of Snail expression during development (77).

A diagram of the current model of Snail expression in EMT is depicted in Figure 2. In epithelial cells, Snail transcription is low. Transient stimulus such as TGF-β induces Snail protein that binds to Snail gene and interrupts the input. This is a consequence of the high expression of E-cadherin in these cells that prevents the stimulation of NF-κB

and perhaps other signalling pathways. In cells where E-cadherin function has been weakened, increases in Snail expression are amplified by the self-stimulatory loop, enabled by the lower repression of NF-κB by E-cadherin. Down-regulation of E-cadherin by Snail further enhances this loop. Self-activation overpasses the limitation imposed by the binding of Snail to its own promoter and leads to a high stimulation in Snail expression. Moreover, increases in NF-κB promote the induction of other mesenchymal genes, such as fibronectin or LEF1, or other transcriptional repressors of CDH1 gene expression, such as Zeb1. Once Zeb1 is induced, Snail expression can be down-regulated without a reversion of the phenotype. This hypothesis would explain the current vision of Snail as a gene required for triggering EMT, but not for maintaining the mesenchymal phenotype (36). Another consequence of this model is that the flow of information from Snail towards E-cadherin is not totally unidirectional. Thus, Snail blocks E-cadherin transcription but also E-cadherin modifies Snail gene expression. Accordingly, we expect that most of the factors that increase E-cadherin protein or function down-regulate Snail gene expression.

Another common theme that comes out from the studies on the signalling pathways controlling EMT and Snail is the existence of multiple points of incidence of a given stimulus. For instance, activation of NF-κB not only increases SNAIL transcription and stability of the protein, but also up-regulates the synthesis of other mesenchymal proteins, such as Lef1 or Fibronectin by direct binding to the promoters of these genes (12). Therefore, the effect on these genes is multiple, since NF-κB activates their transcription both directly and indirectly, up-regulating the expression of their activator Snail. A similar multiple action is also observed in the repression of E-cadherin by Snail. Besides binding its promoter (20, 21), Snail also increases the synthesis of the repressors Zeb1 and Zeb2 (78, 79) and blocks the synthesis of activators of E-cadherin transcription, such as the vitamin D receptor (80). Finally, TGF-β also acts on Snail at least in two steps, since it increases its transcription (36, 62) but is also required for the maximal repression of E-cadherin through the recruitment of SMAD proteins to the CDH1 promoter (45). Therefore, it is possible to envision the EMT as a cooperative process in which, when the stimulus reaches a certain threshold and E-Cadherin levels are down-regulated, a number of factors (NF-κB, Snail, Zeb proteins) are successively co-opted to amplify the signal and induce the massive changes in gene expression that characterize this transition.

Although frequently neglected, Snail protein stability is also relevant in the control of expression. Several studies indicate that stability of Snail protein is also controlled by stimuli involved in EMT. For instance, Snail phosphorylation in the central domain by GSK-3β and subsequent degradation is modulated by Wnt ligands (81). Snail protein stability is also increased in hypoxia, a condition that does not up-regulate SNAIL mRNA (53); this enhanced protein stabilization is associated to the down-regulation of Fbxl14, a ubiquitin ligase involved in Snail degradation. Increased Snail protein stability and down-regulated Fbxl14 expression induced by hypoxia are dependent on the expression of Twist, another transcriptional factor involved in EMT that contrarily to Snail does not directly inhibit E-cadherin transcription (82). These results also indicate that Snail cooperates with other factors in triggering EMT.

Protein stability is also controlled by inflammatory cytokines (83) that, through the activation of NF-κB and the synthesis of COP9 signalosome 2 (CSN2) protein, prevent the interaction of Snail protein with β-TrcP1 and probably also with Fbxl14. These results indicate that Snail protein stability is finely regulated, in some cases by the same stimuli leading to increased transcription, such as those activating NF-κB. However, it is also possible that some conditions would only act on the regulation of protein degradation.

It is likely that other ubiquitin ligases, apart from β-TrCP1 and Fbxl14, also participate in the regulation of Snail protein expression, since a low stability is observed for this protein in most cell lines. We speculate that the activity of these ubiquitin ligases will also be affected in conditions that induce a sustained Snail expression, such as stimulation by TGF-β, inflammatory cytokines or hypoxia, to adequately translate the augment in Snail mRNA, when observed, into a significant up-regulation of Snail protein. In any case, and considering that Snail mRNA and protein levels do not necessarily correlate, studies on the relevance of Snail expression in certain physiological and pathological conditions must be concluded upon the analysis of Snail protein expression and not RNA.

6. EMT during breast development.

Breast development is characterized by several distinct phases involving cell migration and differentiation. During embryogenesis, a mass of epithelial cells condenses along two mammary lines, resting on a strip of condensed mesenchymal cells. Following

epithelial-mesenchymal interactions, epithelial masses grow inward through the mesenchymal layer, forming epithelial buds. Along the dramatic cell reorganization, an epithelial cord emerges and, in mouse embryo, gives rise to the unique lactiferous sinus and to secondary sprouts. At puberty, following hormonal signaling, secondary and tertiary sprouts grow and invade the mammary fat pad through structures called terminal end buds (TEB) in mouse. The basal layer of cells at the front of these structures are called cap cells; they exhibit a partial EMT phenotype, including a re-organization of cell-cell adhesion structures, expression of specific cadherins (P-cadherin versus E-cadherin expressed in luminal cells), switch in cytokeratin expression pattern and invasive properties, resulting in an average 5-10 μM/h migratory speed along the 7-week long tubulogenesis period. Interestingly, cell-cell adhesion is required for the bud growth, involving E and P-cadherins. Interfering with cell-cell adhesion actually inhibits the TEB migration by inducing TEB cell dissociation (84). Migration is linked to proliferative and proteolytic (matrix metalloproteinases) activities. No cytoplasmic extensions such as filopodia are seen at the migrating front. As found during cutaneous wound healing, cell proliferation is compatible with a partial EMT phenotype. Conversely, apoptosis is seen in cells located at the rear aspect of the TEB, involving cells that are not engaged in partial EMT. Several EMT pathways including IGF, Wnt, Notch and TGFβ are activated within the TEBs and appear to control tubulogenesis (85). Interestingly, EMT "master genes" such as Twist and Snail are also found overexpressed in TEBs. Among them, Slug appears to be causally involved in tubulogenesis pattern (Savagner et al, unpublished data). Following mammary fat pad invasion, mammary epithelial cells go through cycles of growth and apoptosis, followed by morphogenetic stages. Each pregnancy involves extensive growth and branching, followed by massive apoptosis and full remodeling of tertiary tubules and terminating alveoli at involution. It is likely that the same pathways, including Snail genes, are involved in these events.

7. EMT in mammary tumors: basal-like and luminal type cancers.

Invasive breast carcinomas are characterized by their strong heterogeneity, reflected in the tumor histology, clinical presentation and response to therapy. Their clinical classification has been based on histological features including the presence of differentiated tubules, proliferation rate (mitotic index) and anisokaryosis, the bases for the Nottingham and Scarff Bloom Richardson grading system (86). Other properties such as hormonal receptor status have been found to correlate with disease progression and are used as markers for diagnostic and prognostic purposes.

Therefore, due to this heterogeneity, it is likely that the contribution of a process like EMT in cancer progression depends on the tumor type. A limitation of the clinical studies is the impossibility to state if an undifferentiated phenotype reflects a lack of differentiation or an active process of EMT during tumor progression. However, a classification of EMT-like phenotypes based on cell-cell adhesion status has been recently proposed, without presumptions about mechanisms responsible for this phenotype. The best case examples in which a complete EMT takes place during tumor progression are carcinosarcomas or metaplastic carcinomas, which represent fewer than 1% of invasive breast carcinomas, but carry a bad prognostic. In these tumors, an epithelial and a mesenchymal compartment can be distinguished based on the expression of, respectively, cytokeratins or vimentin intermediate filaments. Cytogenetic studies strongly suggest that these two compartments originate from a common precursor cell population undergoing a full EMT process giving rise to the mesenchymal component (87). Recent studies show overexpression of Snail and Slug in these tumors, correlating with activation of Akt and β-catenin pathways (88).

A more prevalent mammary tumor, the infiltrating lobular carcinoma, is also characterized by the lack of E-cadherin expression reflecting genomic and epigenetic silencing mechanisms (89, 90). These tumors express significantly high levels of the EMT-related gene Twist but interestingly still express cytokeratins. They provide an interesting case of partial EMT resulting in individualized cells. This phenotype results in a distinct and more insidious mode of invasion using an “indian file” pattern, consisting of an alignment of 3 to 10 cells following but not adhering to each other. Unfortunately, these tumors that represent 10 to 15% of invasive breast carcinomas tend to be detected late during tumor progression, resulting in a poorer prognosis.

More recently, expression profiling has provided new global approaches. Based on unsupervised clustering, most studies sort breast tumors in five groups including basal-like, ERBB2 over-expressing, luminal A and B and normal–like tumors (91, 92). Expression profiles and signatures characterize these groups, reflecting histological features and tumor phenotype. However, their definite identity is still an ongoing process. Most of the studies have identified a group called the basal-like group. This group appears to be heterogeneous, probably encompassing several subtypes, such as “triple-negative (ER, PR, HER2/ErbB2) tumors”. Tumor cells in this group present a phenotype reminiscent of the elusive stem cell profile described for mammary gland. Several authors have suggested that basal-like cancers could be generated by

mammary stem cells transformed at very early stages of differentiation (93). This observation is also relevant considering the links established between EMT and emergence of stem cell-like cells (3, 4). Several pathways activated along EMT models are also overactive in basal-like carcinomas. These include the oncogenic cMyc pathway, recently reported to activate Snail/GSK3β axis and induce EMT (94, 95). Also the expression of factors of the Snail family has suggested that EMT is controlling basal-like carcinomas progression (96, 97). It should be noticed however that basal-like tumor cells profile is distinct from a post-EMT profile. Indeed, basal-like cells typically express significant levels of vimentin, cytokeratins 5/6 and EGFR resembling an undifferentiated (basal) phenotype, but also show typical epithelial markers such as CK8/18 and E-cadherin (91). Overall, basal-like carcinomas are associated with poor relapse-free and survival. Interestingly, regional differences have been noted in this pattern between North American and Asian clinical studies (98). Another tumor group called the normal-like tumors is also characterized by the expression of some markers and the activation of pathways characteristic of early differentiation (99). In both cases, it is tempting to suggest that tumor cell phenotype could reflect a low differentiation level compared to the original transformed tumor cell. Alternatively, the initial transformation process could include a de-differentiation stage, possibly an EMT situation, considering that EMT-related pathways are found to be activated during transformation and tumor progression.

Several EMT "master genes" have been suggested to play a role in this process. Among them, Twist and Snail genes emerge as promising candidates. The role of Twist in mammary cancer metastasis has been demonstrated in mouse and human tumors, with a specific overexpression in invasive lobular carcinoma (82). Fuction of Twist in early transformation stages has been studied in mammary epithelial cells linked to escaping senescence and repressing differentiation by interfering with Rb and P53 pathways. Interestingly, this pattern includes EMT induction and early cell dissemination (100, 101). The association between early transformation and dissemination suggests that metastatic progression could be a very early event rather than a slow process resulting from clonal selection, as suggested in classic models.

The other prevalent EMT "master genes" are members of the Snail family of transcription. A significant number of publications suggest an overexpression of Snail members linked to tumor aggressiveness (102). One recurrent problem has been the lack of reliable antibodies for immunolocalization. This problem has been solved

recently for Snail. Our studies have clearly demonstrated Snail expression in human mammary carcinoma cells, but also in stromal cells (103, 104). Interestingly, this expression is not always linked to E-cadherin down-regulation in epithelial cells, suggesting the lack of Snail co-repressors in these samples. Slug was also found over-expressed in mammary carcinomas, including the previously mentioned basal-like carcinomas (96, 97). Similarly to Snail, *in situ* hybridization studies indicated that Slug expression is not linked to E-cadherin down-regulation although it remains to be determined if Slug RNA and protein levels correlate (102). A significant number of tumor stroma cells were also found to express Slug. Finally, a recent publication using transplantations in humanized mouse mammary gland found Slug among effectors of the Wnt pathway. In this basal-like carcinoma model, a lung metastasis signature was used to identify the Wnt pathway role for self-renewal and proliferation of tumor cells, linking once more EMT, stemness and seeding capacity in human mammary tumor cells (100).

8. Lessons from mouse mammary tumor models: EMT can be linked to specific oncogenic pathways.

Mouse models have been used to decipher the links between cell phenotype, EMT and oncogenic pathways. A recent expression-profiling analysis has established interesting links between mammary-specific tumor promoting pathways and resultant phenotype and dominant active pathways (105). These profiles recall human classification, with notable differences. Basal-like, normal-like, luminal-like and mixed phenotype groups were identified by a clustering based on an 866 gene signature designed by unsupervised tumor sample clustering. These group phenotypes were preferentially found in tumor models including MMTV-Wnt1, p53 null transplant, DMBA, BRCA1xP53/irradiated (basal-like phenotype); MMTV-Neu, MMTV-PyMT, WAP-Myc, Wap-Int 3 (luminal-like phenotype); WAP-Tag, WAP-T121 (mixed phenotype). These phenotypes appear to result from distinct inductive pathways converging to generate a differentiation status that may also reflect transformed cell origins. Similarly to human mammary basal-like carcinomas, tumor cells from the basal-like group expressed cytokeratin 5 and c-Kit. A separate group called “mesenchymal” was also identified and clearly showed a large scale ongoing EMT in tumors expressing significant amounts of vimentin and Snail among other EMT-related genes. These tumors were mostly composed of dissociated cells. Slug was also found to be over-expressed in this group and in basal-like tumors, as reported for human mammary basal-like carcinomas. This work suggests that EMT-like phenotype *in vivo* can result from oncogenic controlled activation. This was more clearly demonstrated in an intricate

mouse model using MMTV-Cre and FSP-Cre (fibroblast specific protein) strains expressing LacZ under the control of an epithelial or mesenchymal-specific promoter (106). These strains were intercrossed with mouse mammary tumor models: WAP-Myc, MMTV-Neu, and MMTV-PyMT. LacZ expression was monitored in heterozygous mice and reflected MMTV or FSP promoter activity and therefore a marker for the cell origin. Mice from MMTV-Cre/WAP-Myc showed a high expression of LacZ, indicating a strong MMTV promoter activation within most tumor cells, but also in mesenchymal-like cells located outside the histological tumor border. This unequivocal staining illustrated an extensive EMT process affecting a significant proportion of tumor cells. In this model, a good proportion of the peritumoral stroma was actually generated by EMT from the original mouse mammary epithelial cells. Interestingly, no such epithelial-derived mesenchymal cells could be described when crossing MMTV-Myc with MMTV-Neu, or MMTV-PyMT tumor models. This work represents a clear-cut demonstration of an EMT process involved in tumor progression and, in addition to previous work (107), also stresses the specific role of some oncogenic pathways such as Myc and Ras in triggering an EMT process *in vivo* (Figure 3). This is reminiscent from the role of the Myc pathway mentioned before in human basal-like mammary carcinomas. Recent work on p21CIP1 explores links between Myc and Ras–induced tumor progression, EMT and tumor cell stemness (108).

9. EMT in epithelial tumors: a current vision.

As indicated above, both human tumors and mouse models of breast tumorigenesis show evidence of EMT or partial EMT. Besides the modification of the phenotype, EMT also results in the acquisition of other properties involved in carcinoma progression, such as an increased motility, higher resistance to apoptosis and acquisition of stemness properties (see above). All these new capabilities are conferred by ectopic expression of Snail in epithelial cells. For instance, cells that have undergone a Snail-induced EMT present a CD44high/CD24low signature, similar to previously identified cancer stem cells (3). These cells can also originate mammospheres, and differentiate to distinct types of cells (myoepithelial, luminal epithelial). It has also been shown that Snail is required for the synthesis of putative markers of stem cells, such as Aldehyde dehydrogenase 1 (109) and even to induce the expression of stemness-promoting genes, such as Nanog, KLF4 and TCF-4 (110). Probably as a consequence of the acquisition of these properties, the presence of Snail has been associated to higher recurrence in a murine model of breast cancer (111). Therefore, Snail expression will provide epithelial cells with the characteristics of migrating stem cells facilitating the growth and dissemination of epithelial tumors (112).

A question that remains to be answered is the requirement of E-cadherin down-regulation in the Snail effects beyond EMT; thus if the acquisition of stemness or apoptosis resistance is consequence of a *bona-fide* EMT or if these traits are also observed in cells that still express E-cadherin. It is also possible that acquisition of stemness is provided by Snail to some mesenchymal cells, since Snail is not detected in all the cells of this lineage (103). For example, in fibroblasts, where expression of Snail is not constitutive, it is possible that induction of this gene bestows a higher resistance to apoptosis or an unlimited replication potential. Few studies have been performed on the role of Snail in fibroblasts: the only results indicate that Snail expression is essential to induce metalloproteases, which in turn are required for invasion and migration (113, 114).

The induction of Snail expression and EMT can be achieved *in vitro* by over-stimulation of several oncogenes such as Ras or Erb2. However, the best inducers of this process are signals not necessarily associated with tumor growth such as TGF-β or TNF-α. In the case of TGF-β, this cytokine might be a product of the cancer activated fibroblasts present in the tumoral stroma (115) and contribute together with intrinsic signals to the induction of an EMT in tumor cells. Actually, the presence of Snail in the stroma has been reported to be associated with a bad prognosis of colon tumors (104). Besides the possibility that these Snail positive cells correspond to tumor cells that have already undergone an EMT it is also feasible that Snail can provide stromal fibroblasts with a higher capability of inducing migration in tumor cells. In any case, the dependence on tumoral stromal cells for EMT would suggest that cells placed in the tumor-stroma interface would be the most likely to undergo this conversion.

Other signals are also relevant for EMT in tumor cells. Inflammatory signals might also cause it through the coordinated stimulation of Snail and NF-κB (see above). EMT can also be the response of tumoral cells to a highly stressed environment, such as the one originated by hypoxia. In both cases, the tumoral cell might have been doing an illegitimate use of a cellular program initially designed to replenish tissue damaged either by a wound or by low-oxygen. This program would require the reinstatement of stem cell characteristics and the migration to the adequate location.

In conclusion, the concept of EMT has been very fruitful in emphasizing new pathways controlling cell fate and tissue morphogenesis. Particularly, the Snail family has being revealed as a powerful regulator of cell phenotype, also involved in apparently

unrelated cell processes such as apoptosis and acquisition of stemness properties. *In vivo* studies show functional links between these processes along developmental stages, stress response and unfortunately carcinoma progression. Although many questions remain to be answered the remarkable advances made during these last years in the description of the mechanism controlling EMT open new hopes on the use of inhibitors of this process as antitumoral drugs, alone or in combination with other compounds targeting the more sensitive epithelial cells (116, 117).

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Figure legends

Figure 1. Structure of Snail protein. The figure shows a diagram depicting the different domains of Snail protein and the interactors binding to each of them. Phosphorylation sites are indicated as dots: black, if they stimulate Snail action; red, if they inhibit it. The arrow initiating at LOX protein labels the Snail1 amino acids putatively modified by this enzyme.

Figure 2. Self-regulation of Snail expression. In epithelial cells with high adherens junction-mediated adhesion (left), E-cadherin blocks NF-κB activation and translocation to the nucleus. Stimuli, such as TGF-β, inducing SNAIL gene transcription are limited by the inhibitory loop created by Snail protein that binds to SNAIL and EGR1 promoters, down-regulating SNAIL promoter activity. In cells with lower E-cadherin activity (right), Snail induction as consequence of the action of TGF-β or additional signals enhances NF-κB translocation to the nucleus and the stimulation of mesenchymal genes, among them Snail itself. This stimulatory loop is further stimulated by the inhibition of E-cadherin that enables more NF-κB activation. Consequently, Snail expression is temporarily extended favoring the repression of epithelial genes. Some of these Snail target proteins or miRNAs might act like E-cadherin, repressing mesenchymal genes. Moreover NF-κB might also stimulate the synthesis of additional repressors of E-cadherin, providing additional points of crosstalk between the two pathways.

Figure 3. EMT activation during breast carcinoma progression and metastasis. Following tumor initiation, a local microenvironment evolves towards the emergence of a tumor mass, resulting in oncogenic pathways activation, local hypoxia conditions, inflammatory conditions and stroma reaction. These events independently converge to activate EMT to some extent, linked to apoptotic resistance and functional stemness, The EMT intensity is reflected by the invasion mode, allowing cohesive migration or individual cell migration. These processes directly contribute to carcinoma progression and metastasis.

Figure 1

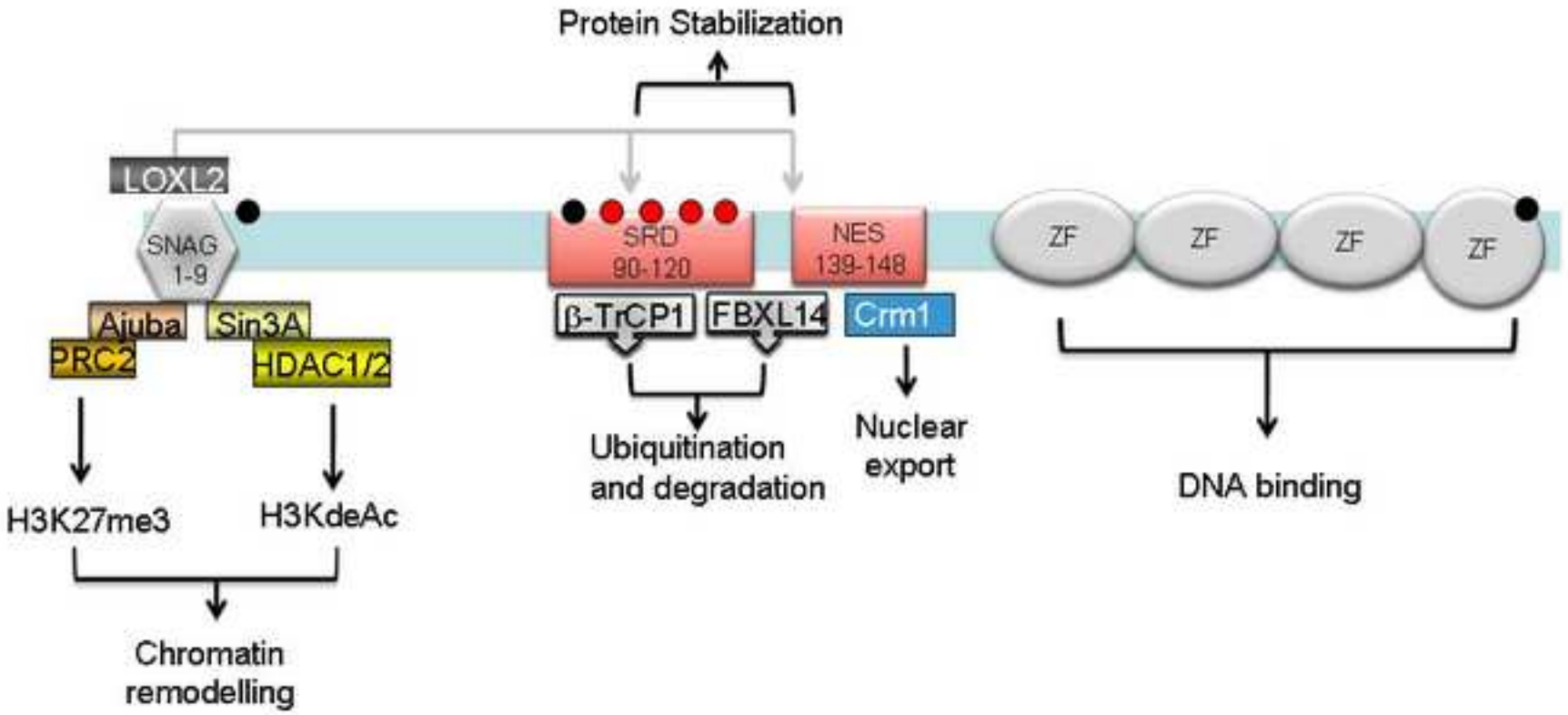

Figure 1

Figure 2

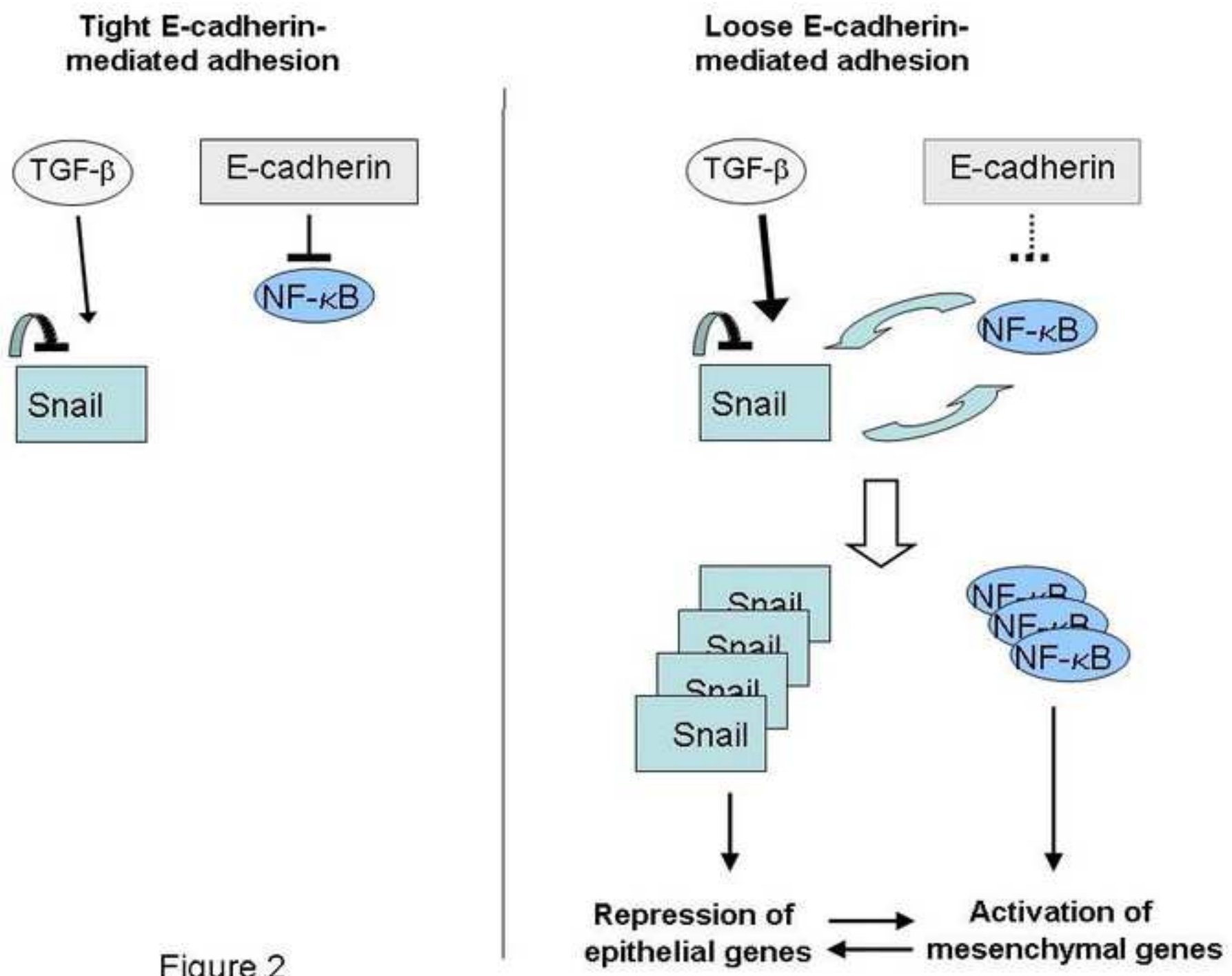

Figure 2

Figure 3
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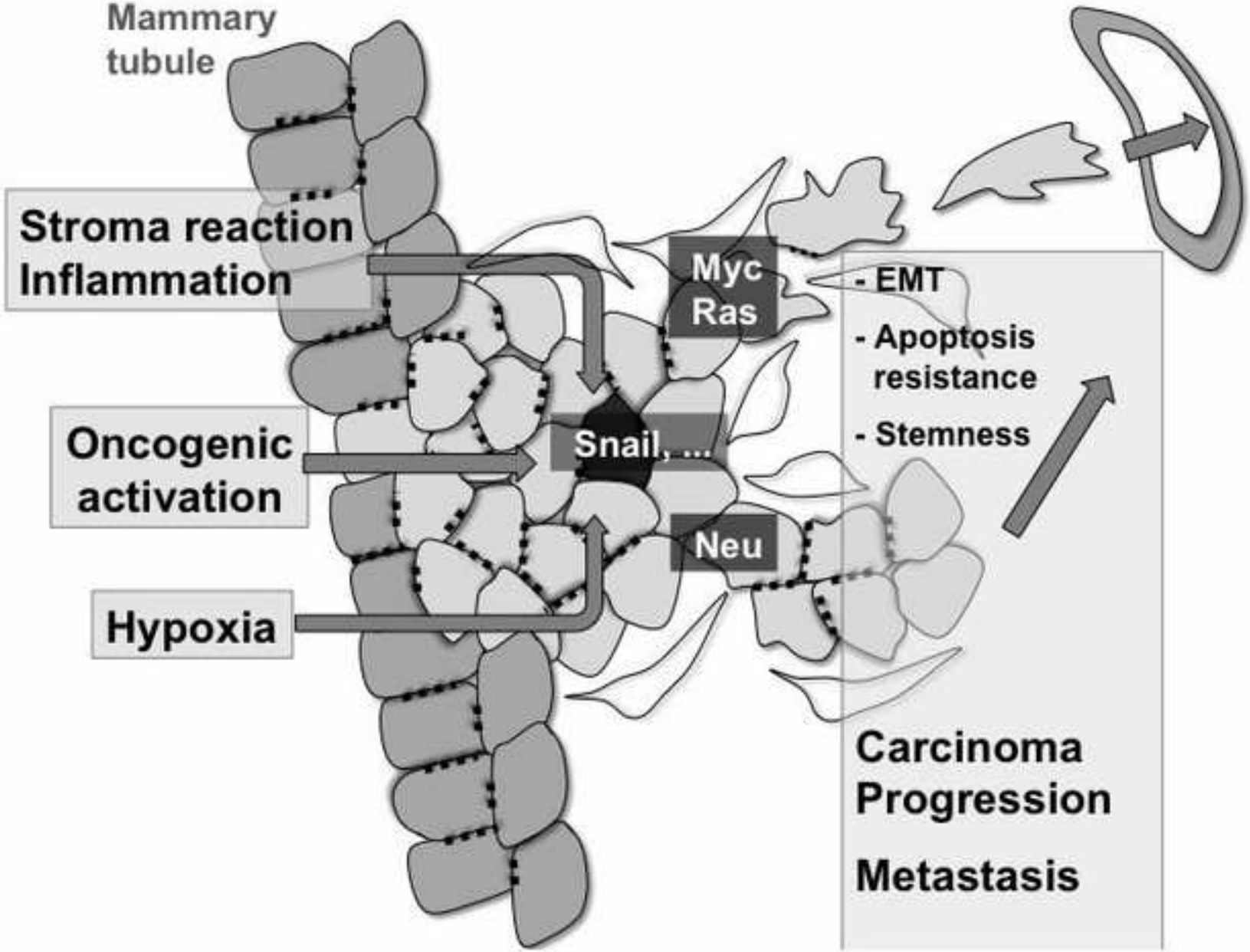

Figure 3

RESPONSES TO THE REVIEWERS

Reviewer #2: Snail family regulation and epithelial mesenchymal transitions in breast cancer progression

In this review, de Herreros et al summarize current knowledge about the Snail family proteins in the context of EMT, and their contributions to tumorigenesis. They place particular emphasis on mammary gland tumorigenesis. While the authors have done an admirable job of critically reviewing the literature, there are a few places in the manuscript that would benefit from minor revision. To this end, I offer the following comments to assist the authors in improving their manuscript:

We really appreciate the reviewer's suggestions and comments. We have proceeded as follows:

a) The manuscript would profit greatly from professional editing to address English syntax, spelling and grammar.

We have received help from several members of our lab with a better command of the English to improve the syntax and grammar and have also corrected all the spelling mistakes.

b) Under section 2, general characteristics of EMT, the authors make several references to 'partial EMT'. It be useful to readers if the authors would define this somewhat more clearly - what molecular, physiological and morphological changes are involved in a 'partial' as opposed to a 'complete' EMT.

In this section we now present a more detailed description of a partial EMT.

c) In section 4, although the title says 'Snail family', the authors have discussed Snail, but have not elaborated very much on the roles of other Snail proteins, such as Slug. I suggest either the authors change the title of this section, or discuss other Snail family members (ie Slug).

We have included two new paragraphs at the end of this section detailing the current knowledge of the biochemistry of other members of the Snail family, Slug and Smuc.

d) Section 5 I particularly liked and commend the authors for their thoughtful treatment of this section.

e) The link between EMT and 'stem-ness' of cancer cells is important and could be discussed in more detail, although the authors touch upon it briefly.

Although it is not the main point of the review, we have followed the reviewer's suggestion and have elaborated further the section linking EMT effectors, particularly Snail, and stemness.

Overall, this is a very well-written overview of Snail, EMT and breast cancer progression. I fully expect that it will be of high interest to the readership of Journal of Mammary Gland Biology and Neoplasia.

Reviewer #3: This review of the role of Snail family proteins in EMT and breast cancer progression has been written, in collaboration, by two labs both well published in the EMT, Snail, and breast cancer fields. The senior authors have extensive experience to draw upon. They are well qualified to be writing such a review. Mostly it is well written with only a few areas of confusing English grammar use (this is not a significant problem at present).

We have edited the review in order to rewrite those parts that might be confusing and to improve the syntax and grammar. We have also checked all the spelling mistakes.

The organization of the review is generally good but I would suggest considering adding a separate section dedicated to breast development and whether Snail family members or EMT has been linked to this. Maybe here the authors can also expand more upon partial EMT, as the current description is not clear. Moreover why are developmental EMT and cancer EMT different and how?

We have followed these two suggestions and expanded point 2, in order to further explain the partial EMT in the context of mammary tubulogenesis. We have also included a new section about EMT in breast development (section 6).

In section three I would suggest considering three other points. First, in tumors with Snail and E-cadherin present, E-cadherin levels need not change rather E-cadherin adhesive function can be altered in cancer. Secondly there is good experimental data that E-cadherin is a bone fide tumor suppressor (Perl et al Nature 1998). Finally I don't think that a "priming" model is necessarily the only way to think about this. It could simply be that other signaling pathways cooperate to influence E-cadherin function when levels persist (see point one above).

We have re-written paragraphs of this section because this is exactly the message we want to convey and since the reviewer did not get, we did not explain it clearly enough. For us "priming" means that cell contacts in epithelial cells are affected by signalling pathways we mention, decreasing E-cadherin adhesive capability. We have also included the very relevant reference indicated by the reviewer.

In section six; mention should be made that other EMT inducers, in addition to Snail, that have been linked to breast cancer, both clinically and experimentally. For example, twist. Extensive detail is not necessary, however, as the review focuses upon the Snail family.

A mention to the results linking Twist with breast cancer is now included in section 7 (former section 6).

Finally, Figure 2 needs to be revised. The present figure implies that there are two, singular, divergent pathways in EMT. One regulates epithelial genes (Snail), the other mesenchymal genes (NF-kB). It is likely more complex than shown. Snail may repress repressors of mesenchymal genes. Snail is likely to also influence miRNA levels as has been seen with ZEB. Thus miRNA targets become critical. And other signals also impinge upon Snail-induced EMT. For example Wnt.

Our idea was to provide a schematic figure showing that Snail and E-cadherin expression is interconnected and controls NF-kB transcriptional activity. The reviewer is correct when he indicates that this figure is a simplification but we have decided to modify it only slightly, for the sake of clarity and in order to better communicate the main message of this figure. In any case, we comment in the text and in the figure legend additional implications and details of our model, such as those indicated by the reviewer.