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## **Roles of the transcription factors snail and slug during mammary morphogenesis and breast carcinoma progression**

**Christophe Côme, Valérie Arnoux, Frédéric Bibeau, Pierre Savagner**

**EMI 0229 INSERM: Génotypes et phénotypes tumoraux, CRLC Val d'Aurelle-  
Paul Lamarque Montpellier, France**

Corresponding author:

P. Savagner

EMI 0229 INSERM: Génotypes et phénotypes tumoraux

Bâtiment de Recherche en Cancérologie

CRLC Val d'Aurelle-Paul Lamarque

34298 Montpellier cedex 5

France

t:33.(0)4 67 61 85 23 / 85 24

f:33.(0)4 67 61 30 41

psavagner@valdorel.fnclcc.fr

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## Abstract

The zinc-finger transcription factors snail and slug are involved in different processes controlling cell differentiation and apoptosis. They appear to be involved in tumor progression. Their putative involvement in mammary gland development has not been specifically examined so far. Slug is expressed at a significant level in normal breast and indirect evidence suggests it could be implicated in tubulogenesis. As an anti-apoptotic agent, it could also protect epithelial cells from death during ductal lumen formation and during breast involution. In breast carcinomas, Snail transcription factors have been linked to tumor progression and invasiveness. Possible mechanisms include repression of E-cadherin gene by snail or slug. However, it is not clear how this transcriptional activity is implicated in vivo. Other possible mechanisms involve maintenance of plastic phenotype by slug that could participate in local invasion of ductal carcinomas and interference with apoptotic pathways that could contribute to global tumor growth and radioresistance. These processes probably also involve interactions with estrogen, EGF or c-kit pathways.

**Keywords:** snail, slug, mammary carcinoma, E-cadherin, invasiveness.

**Abbreviations:** CREaro, aromatase specific CRE; E-cad, E-cadherin; EGFR, epidermal growth factor receptor; EMT, epithelial-mesenchymal transition; HDAC, histone deacetylase; HMEC, human mammary epithelial cells; IDC, invasive ductal carcinoma; ILC, infiltrating lobular carcinoma; TEB, terminal end buds.

## Introduction

***Snail family of transcription factors.*** The first member of the Snail transcription factor family was discovered twenty years ago in *Drosophila*. Since then, homologs have been identified in most animal groups, including nematodes, amphibians, fish and mammals (1-3). Two main families can be distinguished: the Snail family, including snail and slug, and the Scratch family (4).

These factors are mostly known to act as transcriptional repressors. Four to six C2H2 zinc-fingers bind with specific activity the E-box DNA-binding sequence CAGGTG. This motif is common to basic helix-loop-helix transcription factors and therefore is neither restricted nor specific to the Snail family. In mammalian cells, repression activity requires the fingers region and a SNAG domain located in the N-terminal region, typical of the Snail family (4). Some members of the family, such as *Drosophila* snail, lack this SNAG domain. In this case, transcriptional repression results from snail interaction with the co-repressor CtBP (5).

Snail family transcription factors have been implicated in various functions during embryonic development and adult life, including mesoderm differentiation, neural crest formation, left-right asymmetry, neural development, apoptosis, cell division, endoreduplication (4). They may be involved in tumorigenesis. They appear to control a more general phenomenon, the epithelial-mesenchymal transition (EMT) during which epithelial cells lose their cell-cell junctions and acquire mesenchymal characteristics, leading to migratory abilities (6,7).

*Drosophila* genome includes four Snail genes: snail, escargot, wormiu and scratch, mostly implicated in developmental stages, during neurogenesis, mesoderm differentiation, gastrulation and wing development. These four genes act also during

central nervous system development when snail, wormiu and escargot expression territories can overlap (2). This may reflect the physical proximity of snail, escargot and wormiu genes on the second chromosome.

Few direct transcriptional targets have been described for these transcription factors. They include transmembrane adhesion molecule E-cadherin (E-cad) (8-14), aromatase (15), aggrecan, collagen II (16), and Na,K-ATPase  $\beta$ 1-subunit (17). Slug and snail transcriptional repression could be mediated by histone deacetylases (HDACs) recruitment (2). Peinado and colleagues (18) found that E-cad downregulation by snail is mediated by modification of the chromatin. Using its SNAG domain, the transcription factor forms a molecular complex containing effectors HDAC1, HDAC 2 and mSin3A.

In mice, snail is required for early embryogenesis as snail null mutants die at gastrulation stage (19). Conversely, slug null mutants are viable and fertile, even though they express a panel of specific defects (20). In chicken, a functional switch between slug and snail has been described and slug seems to be causally involved in gastrulation (3). In mammals, snail and slug homologs are always both represented in species in which the Snail family has been described. They are overall expressed in the primitive streak, mesoderm, decondensing somites, neural plate, neural crest cells and mesenchymal tissues (1,21).

***Breast development and tumorigenesis.*** In humans, breast development is very similar in male and female embryos but only proceed in females after birth. A rudimentary network of primary ducts invades the fat pad as secondary ducts progressively appear (Fig. 1). This structure is maintained until birth. During childhood, tertiary ducts emerge. However, it is at puberty, in association with ovary-

derived hormones, that the ducts elongate and differentiate into terminal end buds (TEBs). Estrogens are responsible for epithelial cell proliferation, growth of mesenchyme and adipose tissue vascularization. Progesterone is credited for TEBs differentiation into acini. During early pregnancy, ducts extend and cells within the ducts enter apoptosis allowing lumen appearance (Fig. 2A). During late pregnancy, mammary glands enter the lobuloalveolar phase, resulting in final acini differentiation. The alveoli are found in most areas of the fat pad and produce milk proteins and lipids. The breast achieves its full development only after first lactation. After weaning, regression of the mammary gland, called involution, takes place. Apoptosis of alveolar epithelial cells and remodeling of the basement membrane bring back the breast to its prior status. A stronger involution is associated with menopause. Breast pathology is dominated by neoplasms. In the western world, about 10% of women develop breast cancer. Breast tumors are characterized by their considerable histological heterogeneity. Pathogenicity is associated with tumor progression along invasive stages, accompanied with emergence of metastases responsible for fatal evolution in about 25% of identified primary cancers of the breast. Two main histological groups predominate: ductal and lobular carcinomas, representing as a whole 90% of breast cancers. One of the clinical distinction marker between these two types is the transmembrane protein E-cad mentioned above. E-cad repression by snail and slug could be involved in breast tumor progression, as suggested by studies in tumor cell lines. However, recent findings indicate that the situation is more complex in vivo (13,22-24), Magnino, F., Bibeau, F., Côme, C., Theillet, C. and Savagner, P., manuscript submitted).

## 1- Snail and slug involvement during breast development and maturation

Snail or slug expression patterns during breast development have not been specifically described yet. However, some observations suggest they could be implicated during breast development and maturation (Fig. 1). First, human mammary epithelial cells (HMEC) allowed to differentiate on matrigel substrate express slug mRNA at a very significant level, and snail mRNA at a lower level (as found by quantitative PCR; C.Côme, P.Savagner, manuscript in preparation). In addition, a recent publication indicates a modulation of snail expression during menstrual events (25).

### 1. A- Embryogenesis

In the embryo, breast epithelium is derived from ectoderm, whereas snail and slug appear to be mostly expressed in mesenchyme. Slug knockout mice are viable and fertile, suggesting that hormonal balance is established. However, they show significant growth retardation, eye-lid malformations and several problems involving gonad cell populations (20,26). There is no direct evidence for Snail genes involvement in early development of mammary gland.

### 1. B- Post-natal life

***Tubule differentiation.*** Breast development is controlled by breast hormones. Since slug null-mutant mice are fertile, ovarian hormone secretion appears to be normal. In breast tissue, the enzyme aromatase converts androgens into estrogens. Estrogens

are required for normal ductal outgrowth and could implicate slug. In 2001, Okubo and colleagues (27) showed that snail and slug control aromatase expression in breast tissue. As *Escargot* activates tracheal branch fusion in *Drosophila*, they postulate that these transcription factors could be involved in breast ductal network development. But no new indications have been published thus far. We observed that expression of slug, but not snail, is correlated with the expression of differentiated tubes within invasive ductal carcinomas. This suggests that slug could be involved in tubule maintenance or growth in the ductal tumor environment. This expression pattern also suggests this could be the case physiologically during normal tubule formation (Magnino, F., Bibeau, F., Côme, C., Theillet, C. and Savagner, P., manuscript submitted). During ductal development, ducts rise as coherent cellular cords. Lumen space results from controlled apoptosis taking place later within the cord (Fig. 2A). Slug has been described to act as an anti-apoptotic agent (26,28). It is tempting to speculate it could be expressed in peripheral cord epithelial cells to protect them from apoptosis. Slug was also found to promote migratory capacities in hematopoietic cells (29). This function would also be relevant to duct growth. In summary, it appears that slug could be implicated directly during ductal network development.

***Acini differentiation.*** Very little experimental evidence suggests a role for Snail genes during acini differentiation. When primary mammary epithelial cells are cultivated on matrigel, they form acini-like structures that can secrete milk proteins under specific conditions (30-32). We found that HMEC cells grown on matrigel still express significant amounts of slug and E-cad mRNA but very little snail mRNA (C. Côme, P. Savagner, manuscript in preparation). Like in the case of tube formation,



the role of slug in controlling apoptosis could also be exercised during acini differentiation (Fig. 2A). Therefore, slug could also play an active role during the lobuloalveolar phase.

***Involution.*** Involution consists of breast regression after weaning. The mammary gland, which matures during pregnancy and lactation, reverts to a development stage similar to pre-pregnancy. During this involution, a fraction of lobular epithelial cells die, but basal structure is maintained for a possible future pregnancy. Slug anti-apoptotic function could also be involved during this phase (Fig. 2B).

***Menstrual evolution.*** Breast morphology slightly fluctuates during the menstrual cycle. A phase of limited proliferation of epithelial cells occurs at the start of the cycle. Very recently, a study indicated that mesothelial cells cultivated with menstrual effluents upregulate snail and vimentin expression, downregulate E-cad expression and alter cytokeratin expression pattern (25). These morphological changes are characteristic of an EMT, reversible in this case. This report suggests snail expression can be modified in mammary cells by menstrual events.

***Menopause.*** A more important involution occurs after menopause, inducing a limited regression of the breast. Since this stage involves apoptotic events, slug could be downregulated in the regulation of the process.

## **2- Snail, slug involvement in breast cancer progression**

### **2. A- Regulation of E-cadherin by Snail genes**

The transmembrane protein E-cad is the main component of adherens junction, the actin-based cell-cell junction of epithelial cells. E-cad molecules from opposite cells interact through homophilic interactions to connect them. The network of interacting E-cad molecules is stabilized via cytoplasmic links to catenins and, indirectly, actin polymerized microfilaments. Adherens junctions contribute to the establishment of cell polarity and tissue organization. In fact, E-cad-null mice are not viable and die at very early stages of development (33).

In breast, E-cad is expressed by tubule and acini epithelial cells. E-cad regulation during breast cancer progression has been studied for several years. Many carcinoma cells present defects or loss of E-cad expression, correlated with dedifferentiation, lymph node invasion and tumor grade (34-41). Inactivating E-cad with blocking antibodies results in vivo in the disruption of luminal epithelium with the release of individualized cells in the lumen (42). Conversely, maintenance of E-cad in invasive tumors can block the tumor progression in mouse model (43).

E-cad is specifically regulated in the two main breast tumor types mentioned earlier. Invasive ductal carcinomas (IDC) show positive, but heterogeneous expression patterns, with overall persistence of E-cad immunostaining within a specific tumor. In contrast, infiltrating lobular carcinomas (ILC) express no membrane-linked E-cad in all cases and no E-cad protein in most cases (44). Distinct molecular mechanisms appear to be involved. Somatic mutations in E-cad gene have been described in 10 to 56% of lobular breast tumors models (45-48). Oppositely, no mutations were described in ductal breast carcinomas (48). The fact that most lobular carcinomas

express no more E-cad indicates additional mechanisms may be involved in E-cad downregulation.

The involvement of reversible epigenetic mechanisms is suggested by the expression of E-cad in metastases originating from E-cad negative carcinoma (24,49). Hypermethylation of E-cad promoter has been reported to correlate with loss of E-cad expression in vivo in breast carcinomas (22,50,51). This epigenetic plasticity also reflects the complex transcriptional control of E-cad. Basal activity appears to involve Rb, c-Myc and AP-2 (52,53). Other factors regulating E-cad promoter include TGF $\beta$  (54), WT1 during embryogenesis (55), ILK within the  $\beta$  catenin/LEF pathway (56), Erb-B2, a proto-oncogene frequently overexpressed in breast tumors (57), and zinc-fingers factors SIP1, snail and slug, both targeting E2-boxes in E-cad promoters (8,9,58).

In breast cancers, snail and slug have been found in several tumor cell lines and in some mouse models to correlate negatively with E-cad expression levels, shedding new light on the possible involvement of these factors in tumorigenesis (13,22,23), Magnino, F., Bibeau, F., Côme, C., Theillet, C. and Savagner, P., manuscript submitted). However, E-cad is generally expressed in the main type of breast cancers, the IDCs. The overall level of E-cad mRNA expression is not downregulated significantly in IDCs as compared to normal breast samples (Magnino, F., Bibeau, F., Côme, C., Theillet, C. and Savagner, P., manuscript submitted). These findings were confirmed at protein level by several authors (23,24). About 50% of the 22 IDCs studied in the first report and more that 70% of the corresponding metastases were found to express normal levels of E-cad. It is an important demonstration that E-cad repression could be only transient during IDC progression

to metastatic stages.

E-cad repression could also be limited to specific areas within a tumor, providing an escape mechanism for invasive or metastatic cells. These areas could represent invasive fronts linked to local progression, in connection with increased invasiveness associated with mesenchymal phenotype (Fig. 3). Supporting this view, snail has been implicated in MMP2 induction in squamous cell lines (59), suggesting a role in promoting invasiveness. Detached cells could later re-express E-cad, as they move along the chain of events leading to an established metastasis, providing anchorage and growth opportunities (Fig. 3). Unfortunately, it is difficult to validate this hypothesis in vivo in pathological samples. The notion of invasive front is mostly relevant to local progression when metastatic progression is more likely to involve the state of local vascularization. Our unpublished observation show that normal HMEC express high amounts of slug and E-cad mRNA, but very little of snail (Magnino, F., Bibeau, F., Côme, C., Theillet, C. and Savagner, P., manuscript submitted). This is also observed in a few breast cell lines in which slug or snail and E-cad are co-expressed (13), Magnino, F., Bibeau, F., Côme, C., Theillet, C. and Savagner, P., manuscript submitted). In our observations, these high levels of slug and E-cad coincided with a highly motile behavior and the ability to differentiate in vitro, depending on culture conditions, demonstrating that E-cad expression, even at the protein level, is not incompatible with cell motility. In fact, we found that E-cadherin mRNA level was actually positively correlated with slug or snail mRNA level on a sampling of 60 IDCs.

Snail and slug transcriptional activity could also be combined with other mechanisms. Snail impact on E-cad repression was evaluated compared to hypermethylation and

loss of heterozygosity in 81 sporadic IDCs (22). On 67 tumors, 37,3% present loss of heterozygosity. Cheng and colleagues indicate a current increase of snail expression and hypermethylation, associated with E-cad repression. They hypothesize that these two phenomena could act in concert to downregulate E-cad expression.

In lobular carcinomas, mammary epithelial cells go through a typical EMT, but this may not involve snail or slug since genomic events appear to be repressing E-cad expression in about half of the cases. However, Kowalski and colleagues found that when none of a sampling of nine primary ILC expressed normal E-cad levels, two of the derived metastases recovered a full and normal expression of E-cad. This suggests that E-cad repression was, in some cases, reversible and potentially linked to transcriptional control. No studies so far manage to correlate E-cad genomic regulation and snail or slug expression level. Indeed, no correlation was found between E-cad expression level (significantly downregulated) and Snail transcription factors in ILC (23), Magnino, F., Bibeau, F., Côme, C., Theillet, C. and Savagner, P., manuscript submitted).

## **2. B- Role of Snail genes in metastasis**

In vivo, two studies based on IDCs indicate a possible involvement of slug and snail in breast tumor propensity to develop metastases. The first one showed expression of snail by in situ hybridization in breast tumors presenting metastases (23). More recently, we found, using quantitative RT-PCR, that slug or snail expression levels were significantly higher in IDCs associated with invaded lymph nodes (Magnino, F.,

Bibeau, F., Côme, C., Theillet, C. and Savagner, P., manuscript submitted).

Interestingly, a positive correlation was found between snail and slug expression in these tumors.

Metastasis is a multistep process and requires the expression of novel abilities by cancer cells. Distinct metastasis strategies are used by cancer cells depending on cancer types and organs. EMT induction in cancer cells in vivo could confer them increased invasiveness. However, among human breast cancers, ILCs are characterized by the lack of E-cad expression by the cancer cells, linked to a global EMT. But ILCs do not prove clinically to be more or less aggressive than IDCs. Therefore, it appears that EMT, possibly under the control of snail genes represent only one of the possible invasive strategies selected by cancer cells. In tumor types such as IDCs, that constitute the large majority of human breast cancers, most of the tumor cells appears to be included in relatively coherent cords, tubules or clusters. As opposed to snail, slug could play a distinct role in the maintenance of cell plasticity associated to a limited epithelial differentiation. This activity could result in an increase of local invasion that would contribute indirectly to metastasis emergence (Fig. 3).

## **2. C- Snail and slug connections with the estrogen pathway**

Estradiol is a physiological activator of mammary epithelial cells in vivo. It is required for normal differentiation and acts as a potent mitogen in many breast cancer cell lines by stimulating the G1 to S transition (60). An overexpression of estrogen or estrogen receptor can induce proliferation of cells and make them tumorigenic.

About 70% of breast cancers are estrogen receptor-positive and estrogen dependent (61).

**Aromatase regulation.** Aromatase is an estrogen synthetase and converts androgen to estrogen, a necessary step in the estrogen pathway (27,62). Breast cancer samples express more aromatase than normal breast tissue (63). Aromatase gene regulation is mediated by distinct promoters activated in a tissue-specific manner. In normal breast tissue, promoter I.4 is active whereas in breast cancer cells and adipose stromal cells, the alternate promoters I.3 and II are activated. Snail genes could participate in controlling the promoter I.3 and II in normal tissue (Fig. 4), but would not be active in tumor cells (64). Accordingly, a negative correlation was found between slug and aromatase expression. In estrogen-dependant breast tumors, aromatase overexpression could induce cell proliferation, but more clinical observations are needed to elucidate this pathway since we found significant amount of slug expression in a majority of breast invasive carcinomas (Magnino, F., Bibeau, F., Côme, C., Theillet, C. and Savagner, P., manuscript submitted).

**Snail regulation by MTA3.** Human MTA3, an estrogen-responsive gene, is a component of the histone deacetylase MI-2/NuRD complex in breast epithelial cells (65). Its expression is directly regulated by estrogen signaling, via estrogen receptor. This complex acts as a transcriptional repressor for snail promoter (Fig. 4). Fujita and colleagues observed in estrogen receptor-negative breast tumors a coincidental loss of MTA3 and E-cad accompanied by an overexpression of snail. This downregulation of E-cad could predispose these tumors to invasiveness.

Accordingly, breast cancers expressing MTA3 also tend to express E-cad at a significant level.

## **2. D- Snail and slug connections with the EGF pathway**

Epidermal Growth Factor receptor (EGFR) is expressed by lobular, ductal and myoepithelial cells, and EGF is implicated in growth of normal and transformed HMEC. They are both frequently overexpressed in breast cancers (66). EGF and EGFR expression are correlated with poor prognosis in breast cancer patients. In addition, a negative correlation has been described between EGF/EGFR and estrogen receptor in breast tumors. Estrogen receptor-negative tumors expressing significant amounts of EGF/EGFR have a poorer prognosis than those negative for these two pathways (66).

Several authors (67,68) have described that EGF treatment of carcinoma cells leads to a partial EMT, implicating Snail genes expression and in some cases E-cad inhibition. In addition, EGF was found to induce E-cad endocytosis through a caveolin-1-mediated process (Fig. 4). The resulting dissociation of the E-cad/ $\beta$ -catenin complex activates specific transcription pathways through the  $\beta$  catenin/LEF pathway, activating the snail gene that in turn would repress E-cad expression (68). Calveolae-mediated E-cad endocytosis is a reversible process that allows E-cad molecules to be partially re-expressed at the membrane level. It is intriguing to suggest this mechanism could take place in migratory tumor cells. Overexpressed EGF would lead to a partial EMT in tumors cells. These cells could become isolated



and migrate to blood and lymphatic circulation. Further re-expression of vacuole-sequestered E-cad could contribute to distal metastasis emergence (Fig. 3).

In addition, radiotherapy-induced apoptosis is less prominent in tumors and cancer cell lines presenting high expression levels of EGFR (69). This could be linked to an anti-apoptotic activity mediated by snail and slug as described in the next section.

## **2. E- Snail and slug connections with SCF/c-kit pathway**

SCF/c-kit pathway is involved in preventing death of hematopoietic cells exposed to lethal irradiation. Constitutive activation of the mutated c-kit receptor has been described in many cancers, including breast carcinomas (70). Interestingly, inactivation of c-kit or slug genes in mice produces similar phenotypes including disruption of melanocyte and hematopoietic lineage survival and development, and increased radiosensitivity (28,29).

Recently, the radioresistance conferred to hematopoietic stem cells by SCF/c-kit signaling pathway was found to be mediated by slug (Fig. 4). Slug-deficient mice are more vulnerable to lethal irradiation than normal mice and suffer a lethal anemia (28). This anemia cannot be rescued with SCF in spite of the normal expression pattern of c-kit. Conversely, slug can rescue letally irradiated c-kit-deficient mice by promoting survival of bone marrow hematopoietic stem cell populations. This potential role in contributing to cell resistance to irradiation is specially relevant to the cancer field since tumor-acquired resistance to irradiation is a major clinical concern.

Direct targets mediating slug radioresistance activity are not identified yet. However, another activity of slug in bone marrow cells was discovered, that may be indirectly related. Slug was found to control cell migration abilities: slug-deficient bone marrow cells were found to migrate very poorly through a reconstituted basement membrane as compared to normal bone marrow cells (29). This unexpected finding is also very relevant to the role observed more generally for slug in maintaining cell plasticity. It remains to be seen if the molecular targets involved in this case are also activated in tumor cells.

## **CONCLUSION**

Snail transcription factors have been implicated in many phases of development, but no specific study has been published thus far regarding mammary gland development, probably due to the lack of antibodies suitable for snail or slug immunolocalization. Indirect evidence suggests some potential roles for slug during breast development and maturation but remain preliminary. More data are available concerning snail and slug involvement during breast tumorigenesis. They underscore the potential role of snail and slug during this process, but also emphasize the complexity of the pathways involved in the distinct breast carcinoma types. Snail and slug appear to demonstrate distinct functions during this process, in connection with the invasiveness and differentiation status of carcinoma cells. Snail and slug could initiate EMT phases in tumor areas, resulting in transcriptional repression of E-cad and probably other genes. In addition, slug could be involved in maintenance of semi-differentiated epithelial structures within the tumor. Finally, the

estrogen, EGF/EGFR and c-kit pathways can also control snail and slug expression patterns (Fig. 4). At this point, knowledge about the actual localization pattern of snail and slug within mammary tissue and tumors appears to be required to examine further the relevance of the intriguing molecular models reported here.

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## Figure legends

Fig. 1. Snail genes potential involvement during mammary gland development. Snail and slug may play a role during breast post-natal development by regulating aromatase transcription, tubulogenesis process and morphogenetic apoptotic phases (see also Fig. 2). HMEC: human mammary epithelial cells; TEB: terminal end buds

Fig. 2. Hypothetical roles for slug during breast evolution. A. During duct differentiation, cells within the differentiating filled tubules enter apoptosis, generating the free luminal space. Downregulation of slug in these cells could promote localized apoptosis. Slug expression in parietal HMEC could delineate a territory for surviving cells, defining the tube lining. B. During pregnancy and lactation phases, alveoli differentiate and become enlarged with multiple milk secretory vesicles. After weaning, a large proportion of these cells go through apoptosis to restore the breast to its prepregnant status (see Fig. 1). Expression of slug could characterize the cell population eluding apoptosis and giving rise to the next generation of functional acini. HMEC: human mammary epithelial cells

Fig. 3. Snail genes involvement during breast tumor progression. Local and temporal expression of Snail genes could be involved at distinct stages of breast tumor progression. Possible stages include a) the local progression that involve semi-differentiated tubes or cell cohorts in IDCs, b) individualization of tumor cells by an EMT phase in some ILCs, and c) invasion of blood or lymphatic vessels during metastasis process. Transient downregulation of E-cad could be involved in the process. Better knowledge about the actual expression pattern of Snail genes will be

required to validate these hypothesis. E-cad: E-cadherin; IDC: invasive ductal carcinoma; ILC: infiltrating lobular carcinoma.

Fig. 4. Molecular interaction between Snail genes and estrogen, EGF/EGFR and c-kit pathways during breast tumor progression. A) Estrogen pathway. B) EGF/EGFR and c-kit pathways. Both pathways are frequently up-or downregulated during breast tumor progression. As illustrated here, multiple interactions opportunities with snail, slug and these pathways can be found during normal and tumoral breast differentiation. E-cad: E-cadherin; EGFR: EGF receptor; EMT: epithelial mesenchymal transition; ER: estrogen receptor.

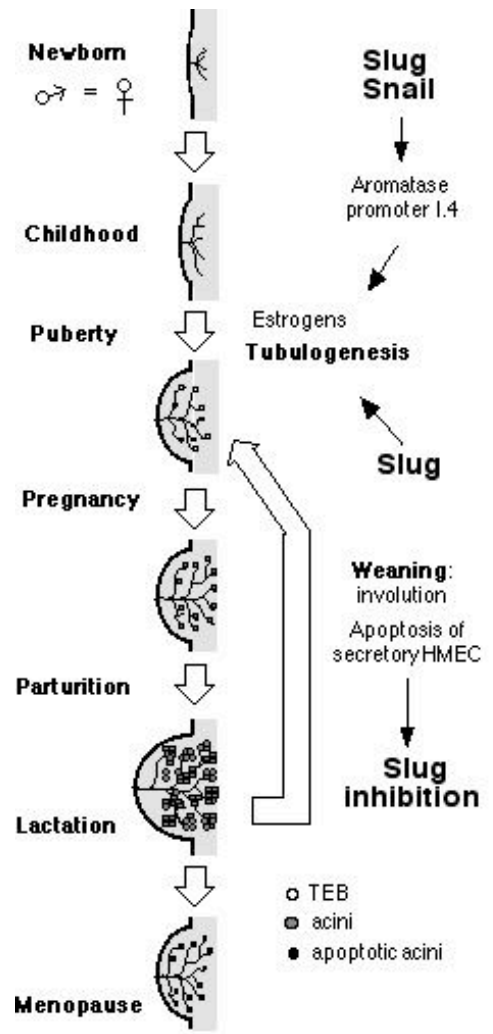
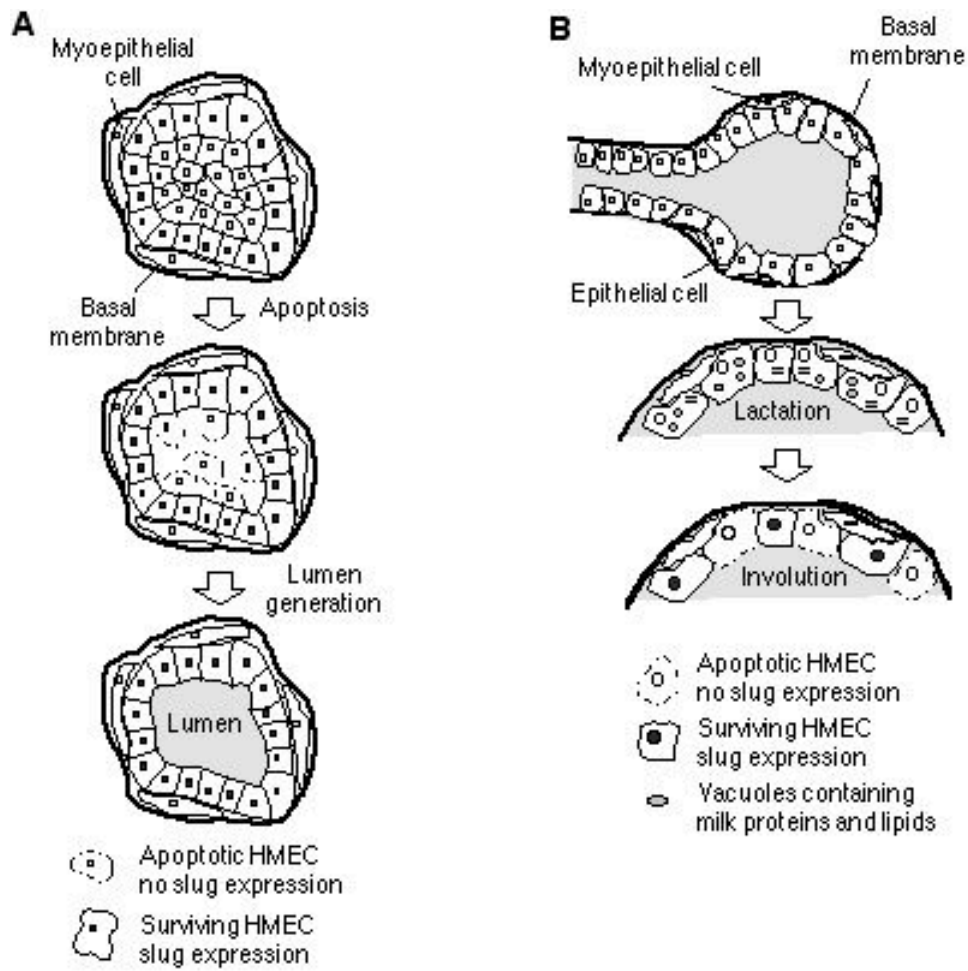


Figure 1





**Figure 2**

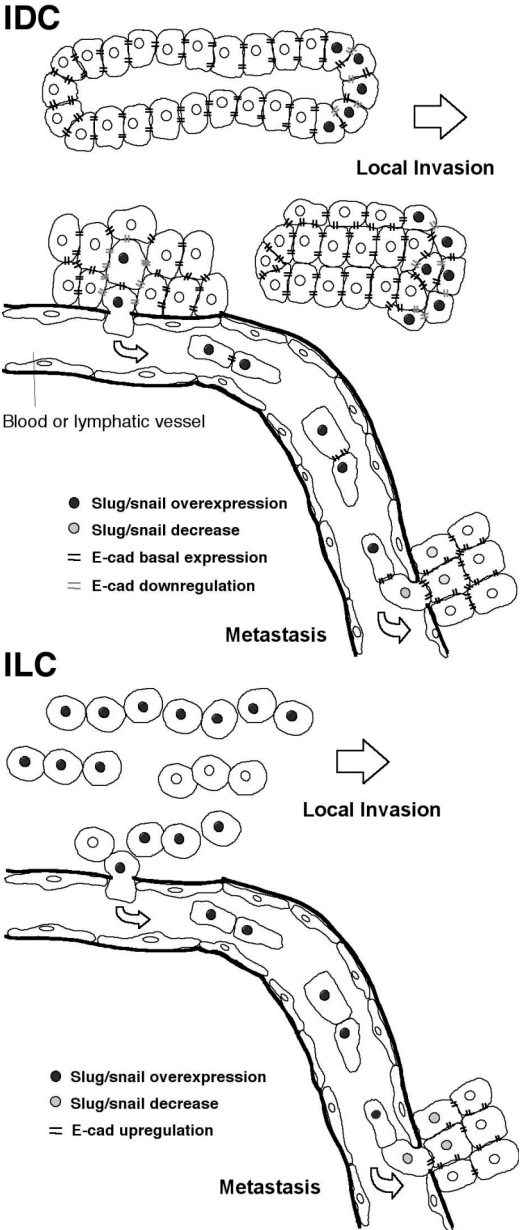
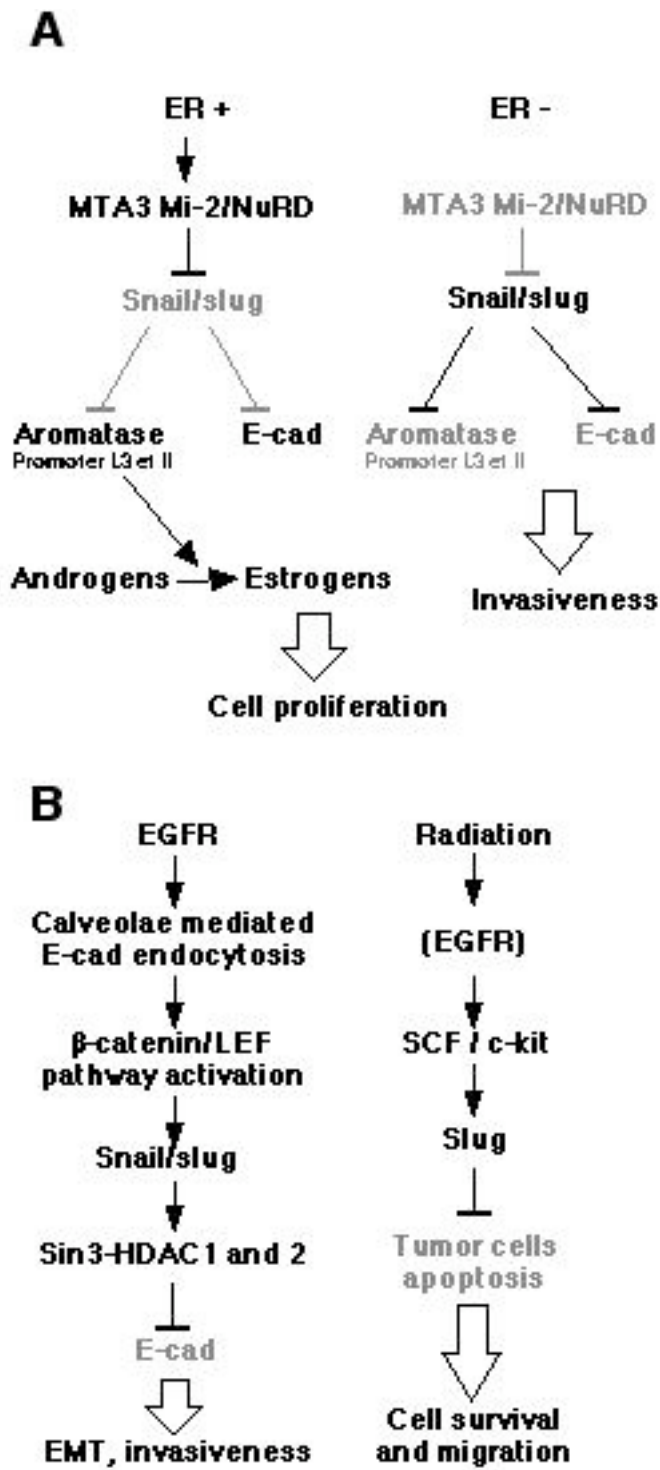


Figure 3



**Figure 4**