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Review Article

Epithelial-mesenchymal transition in cervical carcinoma

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Abstract: During the progression of epithelial cancer, cells usually lose epithelial characteristic features and gain a mesenchymal phenotype. Cervical cancer is a common female malignancy worldwide. Despite the generally good prognosis for early-stage cervical cancer patients, many patients still die as a result of metastasis and recurrence. Epithelial-mesenchymal transition (EMT) has been implicated in the metastasis of primary tumors and provides molecular mechanisms for cervical cancer metastasis. Here we provide an up-to-date overview regarding the program of EMT in cervical cancer. In the stepwise progression of cervical cancer, human papilloma viral proteins contribute to the cell transformation and the conversion of typical epithelial cells to the epithelial carcinoma cells with hybrid epithelial and mesenchymal characteristics. Molecules related to the EMT program of cervical cancer cells are summarized in this review paper. Several soluble factors acting on their cognate receptors stimulate the mesenchymal transition of cervical epithelial cells. Ion transport system as well as cytoskeletal modulators also stimulate the progression of EMT program in cervical carcinoma cells. Transcriptional factors such as Snail, Twist1, Twist2, and six1 homeoproteins are involved in the complicated regulation and cervical cancer metastasis. Among the various signalings associated with EMT program, Snail is a central transcription factor which governs EMT program. In contrast to tumor promoters, several tumor suppressors such as SFRP1/2 and LMX-1A have been reported to suppress tumorigenesis as well as metastatic spread through inhibiting the EMT program.

Keywords: Epithelial-mesenchymal transition, growth factors, transcriptional factors, cervical cancer

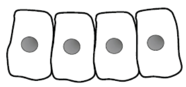
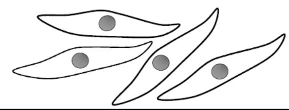
Cervical cancer

Cervical carcinoma is the second most prevalent female cancer worldwide. Squamous cell carcinoma (SCC) accounts for approximate 80% of cervical carcinoma, whereas adenocarcinoma is less common and accounts for 20% of cervical carcinoma. Cervical cancer is mainly contributed by the presence of high-risk human papillomavirus (HPV) oncogene expression [1]. The viral protein products of HPV DNA interact with the anti-oncogenic function of the retinoblastoma and the p53 proteins and inactivate these tumor suppressors in normal keratinocytes. However, not all of those infected by HPV develop cervical cancers; it indicates that factors other than HPV viral proteins also contribute to the progression to cervical cancer [2].

Cervical cancer provides a good model to study the metastatic process due to occurring in a stepwise fashion. The full spectrum of cervical cancer progression includes normal cervix, cervical intraepithelial dysplasia, carcinoma in situ, locally invasive and distant metastatic cancers. When cancer cells become more malignant, they can invade the lymphatic system and spread to distant lymph nodes around the vessels on the pelvic wall. Among these multiple steps, metastatic spread of cancer cells to distant area such as pelvic lymph node is the primary cause of treatment failure and subsequent death in cervical cancer patients. Although Papanicolaou (Pap) screening test is widely used and leads to decline in cervical cancer mortality, many patients with cervical carcinoma still died of metastasis. Therefore, understanding the signaling signatures related to epithelial cell plasticity in the multiple steps of carcinoma progression is an important issue for

Table 1. Epithelial, metastable, and mesenchymal cell phenotypes. Modified from [6].

Epithelial-Mesenchymal Transition (EMT) →

	Epithelial cell	Metastable cell	Mesenchymal cell
Morphology			
Polarity	Apical-basal polarity	Residual polarity	Front-back polarity
Junctions	Tight junction Adherens junction Desmosome	Residual junctions	No junctions
Cytoskeleton	Cortical actin Cytokeratins	Cytokeratins Vimentin	Stress fiber Vimentin Smooth muscle actin
Traits	Stationary	Mixed epithelial and mesenchymal markers	Increased scattering Migration Invasion Anoikis resistance

cervical cancer.

Cancer metastasis

The occurrence of invasion and metastasis is the major cause for most cancer-related deaths. During metastatic progression of carcinoma, polarized epithelial tumor cells gain invasive and migratory characteristics, leave the primary site, invade the basement membrane beneath, intravasate into blood or lymph vessels, transport through the circulation, extravasate from the circulation, disseminate into the secondary site, and grow at the metastatic foci. Epithelial carcinoma cells disseminate from primary tumor sites by using either collective cell migration [3] or single cell migration such as round shape, non-proteolytic amoeboid migration [4] and mesenchymal-type movement [5]. This phenotypic conversion enables tumor cells dissociate from their original tissue and form metastasis in distant organ.

This epithelial cell plasticity caused by breakdown of epithelial cell homeostasis leading to malignant cancer progression has been associated with the loss of epithelial traits and the acquisition of migratory phenotype. In carcinomas, cells awakening the event of mesenchymal transition become motile and increase invasive ability [5]. Hence, the phenotypic transition from epithelial to mesenchymal-like cell state represents one important mechanism for epithelial plasticity and cancer metastasis.

Epithelial-mesenchymal transition (EMT)

Epithelial cells are connected by specialized adhesion complex and have apical-basal polarity. In contrast, mesenchymal cells loosing cell adhesion are spindle-shaped motile cells with front-back cell polarity. Epithelial cells can convert to mesenchymal cells via a multiple-step process referred as epithelial-mesenchymal transition (EMT), which is characterized by dramatic phenotypic changes. The hybrid cell co-expressing epithelial and mesenchymal traits has been introduced as a metastable phenotype [6].

As summarized in **Table 1**, polarized epithelial cells are tightly connected to each other by intercellular junctions composed of tight junction, adherens junction, desmosome, and gap junction. Those junctional complexes function together to prevent cell motility. In the program of mesenchymal transition in epithelial cells include suppression of epithelial adhesion junctions, gain of mesenchymal markers, cytoskeleton reorganization, anoikis resistance, and increased cellular migration and invasiveness. When epithelial cells fully convert to mesenchymal-like phenotype, it benefits the epithelial plasticity.

It has been proposed that three types of EMT exist in physiological and pathological conditions. Type 1 EMT is in the context of developmental processes, type 2 EMT is in inflammation, tissue remodeling, wound healing, and fibrosis, whereas type 3 EMT is in tumor invasion and metastasis [5, 7]. This cellular process is reversible and mesenchymal cells gain epithelial characteristics via mesenchymal-epithelial transition (MET). Incomplete EMT in an epithelial cell may generate a hybrid metastable cell which contains both epithelial and mesenchymal traits, consistent with the existence of carcinoma cells in various tumor system [6].

Epithelial carcinoma cells from benign tumors showing altered epithelial polarity still keep many characteristics of epithelial cells. Whereas cancer progression towards to a malignant, dedifferentiation step, carcinoma cells gain migratory and invasive ability, leave the primary site, invade the beneath basement membrane, intravasate into blood or lymph vessels, transport through the circulation, extravasate from the circulation, disseminate into the secondary site, and grow at the metastatic foci. Epithelial carcinoma cells can adopt single cell mesenchymal-type movement [5] to dissociate from primary tumor sties. This phenotypic conversion enables tumor cells dissociate from their original tissue and form metastases in secondary sites.

Standard histology usually fails to distinguish the mesenchymal-like carcinoma cells from fibroblast-like tumor stroma cells in the tissue sections, however, double staining of epithelial and mesenchymal markers could still define EMT happening in clinical samples. Cancer-associated EMT benefits carcinoma cells for phenotypic plasticity and malignant cancer progression [5, 8]. Moreover, the fine regulation of normal EMT is often disrupted and become exaggerated in cancer cells. Hence, oncogenic EMT signaling pathways facilitate migration and invasion ability of epithelial tumor cells and contribute to other malignant characteristics, such as stem cell traits [9], anti-apoptosis [10], evasion of immune surveillance [11] and chemoresistance [12].

Molecular basis of EMT

Polarized cells are tethered together with well-formed junctional complexes, including tight junctions, adherens junctions, demosomes, and gap junctions. Disruption of adhesion complexes has been thought a preliminary step for losing cell contact in epithelial cells. When epithelial cancer cells gained invasive ability, they are usually associated with reduced expression of the cell-cell adhesion molecules and re-express markers of mesenchymal origin, frequently referred as EMT. The molecular changes in EMT event include (i) Loss of epithelial markers, such as E-cadherin and β-catenin; (ii) de novo expression of mesenchymal-related proteins N-cadherin and fibronectin as well as mesenchymal intermediate filament vimentin; (iii) cytoskeleton rearrangement mediated by Rho small GTPases; (iv) up-regulation and nuclear translocation of transcription factors which govern gene program. Changes in cell morphology and function during the EMT process are accompanied by changes in protein expression profiles, including the loss of the epithelial markers and the de novo expression of mesenchymal markers.

Epithelial cells receive environmental stimuli from growth factors and extracellular matrix proteins through growth factor receptors and integrins, respectively. Previous studies have revealed that EMT can be triggered by interplay of extracellular signals, including extracellular matrix components and soluble growth factors, such as transforming growth factor-beta (TGF-β) and fibroblast growth factor (FGF) families, epidermal growth factor (EGF), insulin-like growth factor (IGF-1) and scatter factor/ hepatocyte growth factor (SF/HGF) in cancer progression [13, 14]. Receptor-mediated signaling in response to these ligands transducer signaling activates intracellular modules and changes cytoskeleton reorganization. Finally, the signaling pathway leads to the activation of nuclear transcriptional regulators, which regulate the global cellular changes on the gene expression level.

In the mesenchymal trans-differentiation process of epithelial cells, downregulation of E-cadherin has been characterized as the major hallmark responsible for the loss of cell-cell contacts in the EMT events. E-cadherin, which is present in mature adherens junctions, is a pivotal molecule maintaining epithelial cell polarity. E-cadherin binds to β-catenin to form a protein complex which links to actin cytoskeleton. E-cadherin has anti-proliferation, anti-invasion, and anti-metastasis functions, and loss of E-cadherin contributes to metastatic dissemination in numerous cancer types [15]. Mechanisms for E-cadherin loss in malignant cancers include genetic mutation, epigenetic silencing, transcription repression and proteolytic processing.

It has been generally regarded that E-cadherin is not expressed in mesenchymal cell types due to the action of transcriptional repressors [16]. Several transcription factors, which are majorly expressed in mesenchymal-like cells, have been implicated in the transcriptional repression of E-cadherin gene (CDH1) and EMT events. The zinc -finger protein family of Snail, Slug, and Smuc; two-handed zinc factors of family of ZEB1 and ZEB2 (also known as Smad-interacting protein-1, SIP-1); and basic helix-loop-helix proteins Twist1, Twist2 and E47 have been demonstrated to repress E-cadherin gene expression and regulate other gene function leading to EMT induction [17]. Among these various transcription factors, Snail plays a central role in regulating EMT program [8].

EMT-related signaling pathways in cervical cancer

How cervical carcinoma cells acquire the ability to invade surrounding tissue is not clear understood, but EMT likely plays a role. Using the expression of epithelial and mesenchymal markers to define the EMT process, clinical tissue observations have shown that mesenchymal transition is involved in the invasion of cervical carcinoma cells and associated with the malignant tumor progression [18, 19]. However, literatures on the roles of EMT and the underlying regulatory signalings in cervical carcinogenesis are limited. Current clinical relevance of mesenchymal transition and established signaling pathways in cervical cancer are summarized and described as follows for detailed information. The categories of EMT activators and repressors are summarized in Table 2 and Figure 1, respectively.

HPV viral proteins

During tumor transformation, HPVs have been regarded as etiologic agents for cervical cancer. HPV16 is one of the high-risk HPVs leading to hyperproliferation. Enforced expression of HPV16 E7 causes molecular changes indicative of a mesenchymal transition in normal human foreskin keratinocytes, indicating that HPV E7 viral protein could mediate EMT in the carcinogenesis of cervical cancer [20]. In a parallel study, many epithelial features were gradually eliminated and some mesenchymal traits were emerged in HPV16-transformed keratinocytes during the very early stage of transformation [21]. These two evidences are consistent with the concept that many carcinoma cells are kinds of metastable cells in the states between epithelial and mesenchymal cell types [6].

Soluble factors and their cognate receptors

Soluble factors are prominent components in the tumor microenvironment and conduct their signaling on tumor cells via cognate receptors. Growth factors have been intensively studied for their effects on EMT. TGF-β1 is the most well-known growth factor involved in the regulation of mesenchymal transition. Chronically stimulated by TGF-β1, cervical cancer SiHa cells undergo mesenchymal transition and are accompanied by increased invasion [22]. Other than TGF-β1, chronic treatment of EGF also could induce EMT in cervical cancer cells, the EMT program is correlated with EGFR overexpression and clinical cervical cancer progression [18]. Moreover, the EGFR-mediated signaling pathway leading to EMT can be modulated by extracellular matrix fibronectin and its cognate receptor α5β1 integrin [18]. On the other hand, aberrant upregulated expression of Notch1 receptor, and its ligand Jagged 1, have been also implicated in cervical carcinoma formation [23]. Notch 1 receptor regulates the EMT response through phosphatidylinositol 3-kinase (PI3K)-dependent signaling in the cervical carcinoma cell lines. The interplay between various soluble factors and their cognate receptors affects the fate of normal cervical epithelial cells as well as

Table 2. Currently known signaling molecules involved in the activation or suppression of EMT program in cervical cancer

EMT regulators	Categories	Example of molecules
Oncoproteins promoting cell transformation	HPV viral proteins	HPV16 E7
Metastasis promoters as EMT activators	Soluble factors	Transformation growth factor (TGF-β) Epidermal growth factor (EGF) Jagged1
	Ion transport system	KCl cotransporter-3 (KCC3)
	Cytoskeletal modulators	Rho-GTPase RhoC Gelsolin
	Transcription factors	Snail Twist1 Twist2 Six1 homeoprotein
Metastasis repressors as EMT suppressors	Secreted factors	Secreted frizzled-related protein (SERP1/2)
	Transcription factors	LMA-1A homeobox protein

cancerous ones.

Ion transport system

Intracellular ion homeostasis is important for regulation of cellular functions. Ion transport system has also been demonstrated contributing to initiation of EMT program in cervical carcinoma cells [24]. KCl cotransporter-3 (KCC3) is the most abundant KCC isoform in primary cervical carcinoma [19], and its expression and activity are enhanced by oncogenic growth factor stimulation [25]. Overexpression of KCC3 and the consequent increased KCl cotransport activity benefit cervical cancer cells in the mesenchymal transition and cancer progression by weakening E-cadherin/β-catenin complex formation. KCC3 overexpression negatively regulates the promoter activity of human E-cadherin gene; meanwhile, it accelerates the proteasome -dependent degradation of β-catenin [19].

Cytoskeletal modulators

Cytoskeletal modulators are important factors contributing to EMT, migration, and invasion. Rho-GTPases have been implicated in mesenchymal migration due to their involvement in dissociation of cell adhesions and cytoskeletal remodeling. For instance, aberrant Rho-GTPase signaling is activated by carbonic anhydrase IX [26], which is a tumor-associated membranous zinc metalloenzyme expressed in various human tumors. Overexpression of carbonic anhydrase IX in cervical carcinoma C33A cells caused cellular morphologic changes and augmented cell motility and invasion [26]. In addition, RhoC has been reported to have contribution to EMT and possibly function as a downstream effector of Notch receptor in cervical carcinoma [27]. Stable expression of RhoC contributes to wound healing, migration, invasion, and tumor formation in CaSki and SiHa cervical carcinoma cell lines. Upon wound healing, elevated expression of fibronectin and formation of actin stress fiber were diminished by individual depletion of Notch1 and RhoC, reflecting the involvement of Notch1 and RhoC in regulating EMT response [27]. In addition, gelsolin, a calcium-activated actin binding protein, is overex-

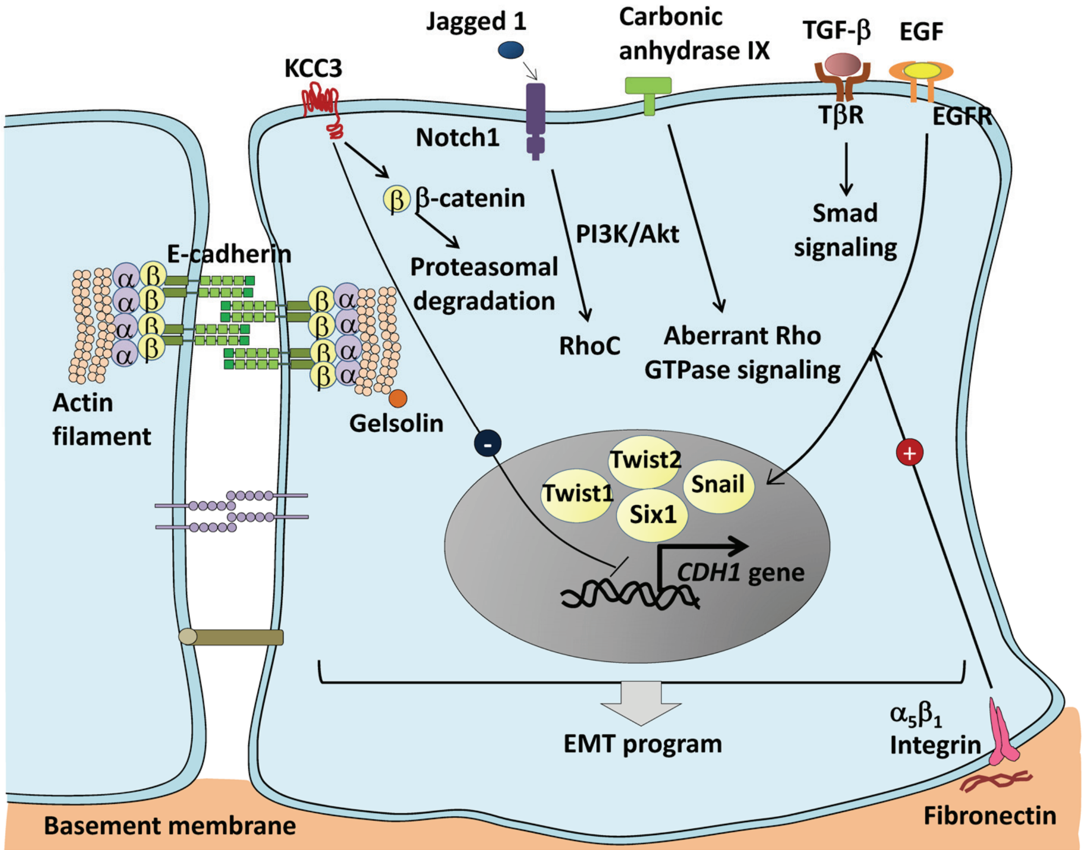


Figure 1. Schematic model of signaling molecules involved in the malignant EMT program of cervical carcinoma cells. Reported signaling molecules related to malignant EMT progression are summarized in this figure. Overexpression of the EGF receptor (EGFR), TGF-β receptor (TβR), and Notch1, respond to the stimulation of their cognate ligands EGF, TGF-β1, and Jagged1, respectively, and transmit signalings to the mesenchymal transition in cervical carcinoma cells. Extracellular signalings from extracellular matrix fibronectin and α5β1 integrin could also modulate the EMT program mediated by EGF. Aberrant signalings from cytoskeletal modulator Rho GTPase regulate the cytoskeletal remodeling and morphological changes in the EMT program. Overexpression of actin-binding protein gelsolin enhances EMT and cell invasion ability. Ion transport system such as potassium chloride co-transporter KCC3 triggers EMT through inhibiting the gene expression of E-cadherin gene (CDH1) and promoting the protein degradation of β-catenin. A panel of transcription factors including Snail, Twist1, Twist2, and Six1 homeoprotein has been reported to regulate the EMT program of cervical carcinoma cells.

pressed in cervical carcinoma. Plasma gelsolin of patients is higher than those of healthy control [28]. Patients with gelsolin high-expression have a lower 5-yeaer overall survival and recurrence-free survival than those with low-expression. Depletion of gelsolin expression in HeLa cells decreased cell migration, reduced MMP-2 and vimentin, and up-regulated E-cadherin, suggesting gelsolin may positively regulate EMT and proteolysis to promote tumor invasion [28]. Together, these evidences support that cytoskeletal modulates indeed contributes to cervical malignancy by controlling the EMT event.

Transcription factors

EMT involves alteration of cell types, several transcription factors have been implicated in the regulation of gene expression related to this transition. Although intensive studies have focused on transcriptional regulators in pathological EMT, little information of transcription factors is addressed using the cervical cancer as a study model. Snail, a zinc-finger transcription regulator, has been reported to be accumulated in the nucleus upon EGF stimulation, and Snail upregulation can be observed in surgical specimen, as shown in **Figure 2** [18]. In addition, en-

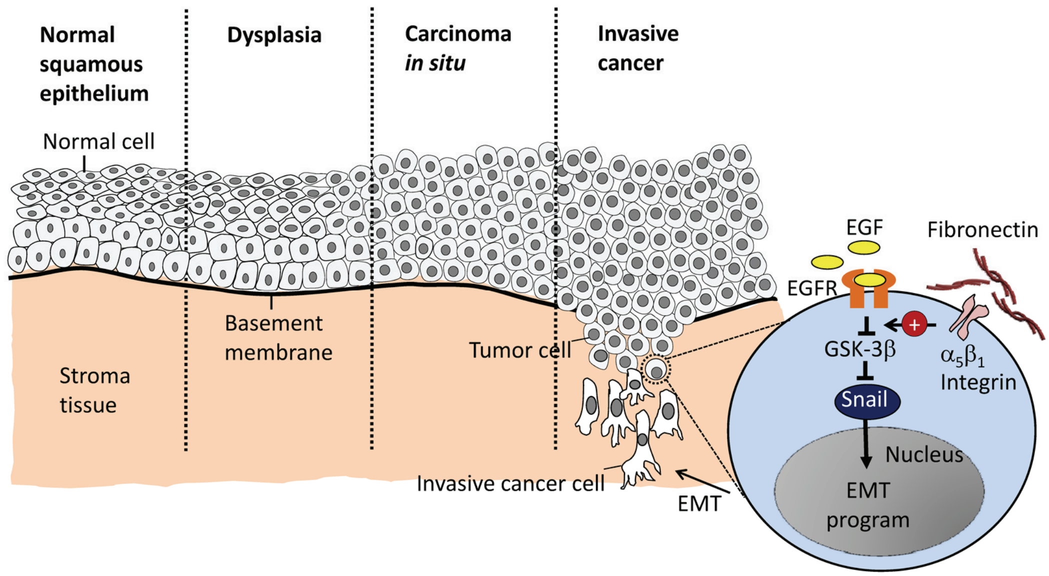


Figure 2. Snail transcription factor is an important transcription factor regulating cervical cancer invasion. The tumor progression of cervical squamous cell carcinoma is developed from normal squamous epithelium, dysplasia, carcinoma in situ, to invasive cancer. Cervical cancerous cells express much more EGFR and are sensitive to EGF stimulation than normal ones. EGF signaling leads to GSK-3β inactivation and then stabilizes the nuclear expression of Snail transcription factor, which regulates the gene expression of the EMT program in the cervical cancer cells. EGF-mediated EMT can be modulated by the signal from extracellular matrix fibronectin and α5β1 integrin. This figure is modified from Clinical Cancer Research 2008; 14: 4743-4750.

forced expression of Twist basic helix-loop-helix proteins in HeLa cells is critical for activation of β-catenin and Akt pathway and maintenance of EMT-associated stem cell-like characters, which have been determined by expression of ALDH1 and CD44 [29]. Twist1 positive expression can predict poor clinical survival rates of cervical cancer patients [30]. Twist2 expression is associated with cervical cancer progression, and it can be used as an indicator for metastasis potential in SCC patients [31]. Moreover, overexpression of Six 1 homeoprotein transcription factor has been reported in various tumors including cervical cancer. It has been reported to promote cancer metastasis and EMT through enhancing TGF-β signaling [32]. The roles of other EMT-related transcription factors and the interplay in this complicated phenomenon deserve more investigation.

Metastasis suppressors

In contrast to tumor promoters, several tumor suppressors have been reported to suppress tumorigenesis as well as metastatic spread through inhibiting the EMT program. Tumor suppressor SFRP1/2 and LMX-1A have been reported to inhibit invasion and metastasis through an incomplete EMT. These tumor suppressor genes are usually inactivated by promoter hypermethylation, hence tumor suppressor gene inactivation lose their ability on protecting cancer from malignant cancer progression. Secreted frizzled-related protein (SFRP) 1 and 2, which function as Wnt antagonists, decrease Wnt signaling and suppresses tumorigenicity [33]. Besides, enforced expression of SFRP1/2 enhances the expression of E-cadherin through inhibiting the expression of Slug, Snail, and Twist, three major transcription factors governing EMT program. Epigenetic silencing of SFRP genes by promoter hypermethylation causes Wnt signaling hyperactivation, contributes to EMT program, and leads to cervical cancer development. In addition, LMX-1A, a LIM homeobox transcriptional factor, suppresses tumor formation and cancer metastasis through inhibition of BMP4 and BMP6 [34].

In summary, HPVs contributes to cell transformation in normal epithelial cells through partially affecting EMT. Cervical carcinoma cells are in a state with concomitant expression of epithelial and mesenchymal markers, similar to

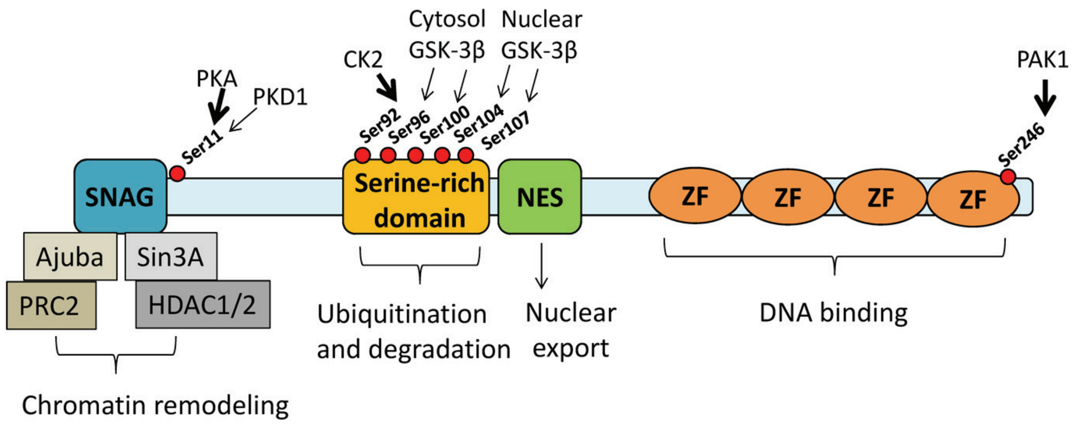


Figure 3. Domain structures and phosphorylation regulation of Snail protein. Snail transcription factor contains four C -terminal zinc finger DNA-binding motifs, a N-terminal SNAG repression domain, and a serine-rich domain and nuclear export sequence (NES) in the central region. SNAG domain repress gene expression through recruiting the complex of Sin3A, histone deacetylase 1 and 2 (HDAC1/2), Ajuba LIM domain protein, and polycomb repressive complex 2 (PRC2). The function and stability of Snail protein are tightly regulated by serine phosphorylation. Several serine phosphorylation residues are noted as the amino acid number in the Snail protein. Serine phosphorylation by cAMP-activated kinase protein kinase A (PKA), casein kinase 2 (CK2), and p21-activated kinase (PAK1) have been reported to enhance the protein stability and transcription regulation ability of Snail protein. In contrast, serine phosphorylation by protein kinase D1 (PKD1) and GSK-3β promotes the nuclear export and degradation of Snail protein. Bold arrow indicates the positive regulation, whereas thin arrow indicates the negative regulation by the indicated molecules. This figure is modified from Journal of Mammary Gland Biolology and Neoplasia 2010; 15: 135-147.

a metastable phenotype between epithelial and mesenchymal cells [6]. Cervical cancer cells undergo EMT event and promote invasion and metastasis in response to the stimulation of overexpressed EMT activators. They are divided in four major groups as follows: (i) receptor-mediated signaling from TGF-β1 [22], EGF and α5β1 integrin [18], and Jagged1-specific Notch signalings [23]; (ii) ion transport system contains KCl cotransporter-3 [19]; (iii) cytoskeletal modulators comprise of gelsolin [28], and Rho C signalings [27]. (iv) EMT-related transcription factors include Six1 homeoprotein [32], Snail [18], and Twist [29]. In contrast, metastasis suppressors such as SFRP1/2 [33] and LMX-1A [34] have also been identified as repressors for EMT program in cervical cancer. The net balance between EMT activators and suppressors determine when to induce mesenchymal transition in cervical carcinoma cells and promote cancer malignancy.

Regulations of Snail transcription factor

Snail family, which includes Snail (Snail-1), Slug (Snail-2), and Smuc (Snail-3), is a group of conserved zinc finger-containing transcription factors [35]. Snail is one of the important Snail family members which inhibit E-cadherin gene transcription and initiate EMT [36, 37]. Nevertheless, more than induction of EMT program, Snail transcription factor functions in many other cellular functions important for cancer biology [8, 38].

Snail transcription factor contains four C-terminal C2-H2 zinc finger DNA-binding motifs and a N-terminal SNAG repression domain [39], as illustrated in **Figure 3**. Snail's nuclear localization signal (NLS) is located within the zinc finger region [40]. Nuclear export sequence (NES) within 139-148 amino acid residues mediates nuclear export of Snail in a Crm1-dependent manner [41].

The zinc finger domain of Snail transcription factor bind to E-box motifs located on the promoter regions of target genes, whereas SNAG domain recruits histone deacetylase 1 and 2 (HDAC1/2) and polycomb repressive complex 2 (PRC2) for chromatin remodeling and further transcription regulation. Besides, nuclear export sequence within 139-148 amino acid residues regulates the subcellular localization of Snail protein.

Genetic deletion of the Snail gene in mice results in embryonic lethality due to exhibit defects in gastrulation and die early before the generation of mesodermal layer [42]. In contrast, the Snail2-null mouse is viable and fertile [43]. It reflects the essential role of Snail in embryonic development.

Snail is the most widely studied transcriptional regulator in the EMT program. Besides having a regulatory roles in EMT, Snail also governs genes related to EMT-independent functions, such as cell survival [44], motility [45], anti-apoptosis [46], immune suppression [47], stem cell properties [9], and chemo-resistance [48]. However, the precise targets of Snail transcription factor involved in these events are currently not so clear.

Snail suppresses transcription by recruiting co-repressors through its SNAG domain [49]. For example, Snail mediates repression of E-cadherin by recruitment of Sin3A/histone deacetylase 1 (HDAC1)/HDAC2 complex [49]. Ajuba LIM domain protein is also a co-repressor for Snail-mediated transcription repression via binding to SNAG domain [50]. Besides, Snail is a transcription factor whose protein stability and activity highly regulated by serine phosphorylation. Phosphoserine binding proteins such as specific 14-3-3s isoforms can form a stabilized ternary transcriptional complex with Snail and Ajuba to influence the endogenous promoter activity of E-cadherin gene [51].

Snail transcription factor binds to the E-box motif found in the promoter regions of target genes through its zinc finger domain. The central core sequence of E-box is 5'-CACCTG-3'[52]. Many epithelial markers, including adherens molecule E-cadherin [36], tight junction molecules claudins/occludin [53], and thrombomodulin [54], have been identified being transcriptionally repressed by Snail. On the other hand, enforced expression of Snail in E-cadherin positive cells also induces the expression of mesenchymal markers including fibronectin and vimentin, however it acts through an indirect mechanism rather than direct transcription regulation [55, 56].

Although Snail has generally been regarded as a transcription repressor to suppress epithelial genes, recent evidences have highlighted the role of Snail as a transcription activator to upregulate gene expression through recruitment of differential transcriptional machinery and chromatin remodeling complex. For example, DNA repair protein excision repair cross-complementation group 1 (ERCC1), whose expression is transcriptionally activated by Snail, is critical for the generation of cisplatin resistance of human head and neck cancer [12]. Additionally, Myosin Va, an unconventional actin-dependent motor involved in cell migration and metastasis of many cancers, is also upregulated by Snail through E-box binding on its promoter [57]. Moreover, Snail associates with Egr1 and SP-1 to transcriptionally activate CDK inhibitor $p15^{INK4b}$ in HepG2 cells upon treatment of tetradecanoyl phorbol acetate (TPA) [58].

The expression level of Snail is tightly regulated at transcriptional, translational and post-translational levels. In the transcriptional control level, activation of SNAIL transcription has been demonstrated by early growth response 1 (Egr1) [59], high mobility group A2 (HMGA2) and Smads [60], or signalings from GSK-3β [61]. In the translational control level, Snail messenger RNA can be translationally activated by Y-box binding protein-1 (YB-1) in a cap-independent manner [62]. In the post-translational control level, phosphorylation regulates the subcellular localization and action of Snail transcription factor [41]. Snail is an unstable protein with a short half-life approximately 25 minutes [63]. Its function is tightly controlled by protein stability and subcellular localization through serine/threonine phosphorylation [41, 63-65], ubiquitination, and degradation [63].

Different phosphorylation motifs have been demonstrated on Snail proteins. Some serine/threonine protein kinases can destabilize and inactivate Snail protein through phosphorylation. Especially, glycogen synthase kinase-3β (GSK-3β) is the well-known central protein kinase controlling Snail stability. Nuclear GSK-3β phosphorylates Snail on serine 104 and 107 and promotes Snail nuclear export. Whereas Snail is in cytoplasm, cytoplasmic GSK-3β further phosphorylates Snail on serine 96 and 100, facilitating Snail binding to β-Trcp1 ubiquitin ligase for ubiquitin-mediated degradation [63]. The GSK-3β-mediated Snail phosphorylation could be removed by the small C-terminal domain phosphatase (SCP) in the nucleus, and then Snail become stabilized [66]. Moreover, protein kinase D1 (PKD1) phosphorylates Snail on serine 11, promotes nuclear export of Snail via 14-3-3δ binding, and suppresses Snail-induced EMT in prostate cancer [65].

In contrast, the subcellular localization and activity of Snail can also be positively regulated by phosphorylation. For instance, p21-activated kinase (PAK1) phosphorylates Snail on serine 246, retains Snail proteins in nucleus, and then augments transcriptional repression functions of Snail [67]. In addition, *in vitro* phosphorylation of serine 11 by cAMP-activated kinase protein kinase A (PKA) is required for Snail-mediated EMT, whereas *in vitro* phosphorylation of serine 92 on Snail by casein kinase-2 (CK2) is required for Snail-mediated cell viability in Madin-Darby canine kidney cells [64]. It raises another question that why respective phosphorylation of Snail serine 11 residue by PKD1 or PKA leads to opposite results, but it is not currently known. Further investigation will be needed to resolve this puzzle.

Stabilization of Snail could also be achieved by other mechanisms. Lysyl oxidase-like 2 (LOXL2) catalyzes the oxidative deamination of lysine 98 and 137 on Snail protein, causes a conformation change that would mask the GSK-3β phosphorylation motif, and prevent degradation [68]. Moreover, NF-κB-COP9 signalosome 2 signaling-mediated Snail stabilization is required for inflammation-induced cell migration and invasion [69]. In summary, Snail expression is tightly controlled especially at the serine phosphorylation level. Understanding the upstream regulatory mechanisms controlling Snail protein stability and transcription function is an important issue in the field of EMT and cancer metastasis.

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