Commentary & View

The role of the transcriptional regulator snail in cell detachment, reattachment and migration

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In order to metastasize, cancer cells must first detach from the primary tumor, migrate, invade through tissues and attach to a second site. The transcription factor snail is an important mediator of epithelial-mesenchymal transitions and is involved in tumor progression. Recent data have provided evidence for a requirement for snail expression in metastatic dissemination. Although very little is known about the molecular mechanisms governing metastatic dissemination, we review the possible roles of snail expression in this process. We also review the regulation of snail expression.

Local tissue invasion represents the first step of the metastatic cascade of carcinomas. Invasion of carcinoma cells requires changes in cell-cell or cell-matrix adhesion, cell polarity and cell migratory properties of the tumor cells. These changes are collectively known as the epithelial-mesenchymal transition (EMT). Downregulation of E-cadherin is an essential event for EMT. Snail is a prominent inducer of EMT and strongly represses E-cadherin expression. Snail also plays important roles in tumor growth and lymph node metastasis of human breast cancer MDA-MB cells. In these cells snail knockdown induces a decrease in proinvasive markers such as matrix metalloproteinase-9 (MMP9) as well as lymph node metastasis. In addition snail-transfected SCC cells showed no laminin-332 synthesis. However, we did not observe enhancement of cell detachment by snail-expressing MDCK and A431 cells. Furthermore, laminin-γ2 knockdown resulted in increased detachment of oral squamous cell carcinoma JHU-022-SCC cells. We also confirmed that the expression of laminin-332 and integrin α6, α3 was reduced in snail-expressing MDCK and A431 cells. However, we did not observe enhancement of cell detachment by laminin-γ2 knockdown in A431 cells or by laminin-α3 knockdown in MDCK cells. Cell adhesion to laminin-332 occurs by binding through α3β1 and α6β4 integrins. Thus, a reduction in integrin α3 and α6, as well as a decrease in laminin-332, could cause detachment of snail-expressing MDCK and A431 cells. In addition to laminin-332, laminin-α5 is also downregulated in snail transfected SCC cells. In contrast, in the same cells, neoexpression of laminin-α4 mRNA and synthesis of laminin-411 (laminin-8) was observed. Laminin-511 is regarded as the most widely expressed laminin and is found in most epithelial BMs. Laminin-511 or -521 (laminin-10/11) interacts with α3β1, α6β1 and α6β4 integrins. Laminin-411 also binds α6β1 integrins. SCC cells potently adhered to laminin-511 whereas adhesion to laminin-411 was minimal. Furthermore, the laminin-α4 chain has been reported to play a role in the detachment of renal carcinoma cells from fibronectin. Laminin-411 decreased adhesion of SCC cells to laminin-511 and to fibronectin by blocking binding sites in the fibronectin molecule. In parental SCC cells integrin α6 is paired with the β4 subunit. When paired with the β4 subunit, integrin α6β4 mediates the formation of desmosomes which link the intermediate filament cytoskeleton to BM laminin-332. Integrin components secreted by the cells or through modulation of the cellular ECM receptors such as integrins. Basement membranes (BM) are sheets of ECM generated by cells at epithelial mesenchymal interfaces. Changes in BM proteins and their cellular receptors are associated with the progression of human carcinomas. The major classes of BM proteins are laminins that are trimers of α, β and γ chains and which bind to a variety of cell surface integrin receptors. To date, 16 different types of laminin trimers have been identified. Modulation of specific forms of laminin or their cell surface receptors may play an important role in EMT and strongly represses E-cadherin expression. Snail also plays important roles in tumor growth and lymph node metastasis of human breast cancer MDA-MB cells. In these cells snail knockdown induces a decrease in proinvasive markers such as matrix metalloproteinase-9 (MMP9) as well as lymph node metastasis. In addition snail-transfected SCC cells showed no laminin-332 synthesis. However, we did not observe enhancement of cell detachment by snail-expressing MDCK and A431 cells. Furthermore, laminin-γ2 knockdown resulted in increased detachment of oral squamous cell carcinoma JHU-022-SCC cells. 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α6β4 also mediates cell adhesion to laminin-511.13 However, in 43A-SNA cells integrin α6 is paired with the β1 subunit.13 Integrin α6β1 is the main receptor for laminin-411. A reduction in integrin α6β4 levels has been shown in snail transfected SCC cells.10 We have detected a loss of integrin β4 expression in snail-expressing cells.5 It has been proposed that a reduction in the level of the integrin β4 subunit allows snail transfected SCC cells to escape from hemidesmosomal contacts and to use the laminin-411 receptor, integrin α6β1, to become motile.13

Collagen IV is another major component of BM. Since normal production and assembly of BM is disrupted during malignant cancer progression it has been suggested that collagen IV α5/α6 chains might protect against rapid cancer progression.18 We have confirmed a reduction in collagen IV in snail-expressing MDCK cells. Detachment of these cells was significantly suppressed when the cells were plated in wells that had been precoated with collagen IV. Thus, a reduction in collagen IV might be at least partly responsible for the increased detachment of MDCK/snail cells.5

Other molecules besides ECM proteins might be involved in the detachment of snail-expressing cells. For example, plasminogen activator inhibitor-1 (PAI-1) has been reported to induce detachment of cells from extracellular matrices by inactivating integrins. The binding of urokinase plasminogen activator (uPA) to its cell surface receptor (uPAR) promotes cell adhesion by increasing the affinity of the receptor for both vitronectin (VN) and integrins. PAI-1 can disrupt uPA-uPAR, uPAR-VN and integrin-VN interactions thereby leading to cell detachment.19 Genetic profiling of snail-expressing MDCK cells revealed enhanced expressions of PAI-1.20 We have also confirmed induction of PAI-1 in snail-expressing cells (unpublished data). Another candidate for the mediation of snail-induced cell detachment is the protein p63. p63, a member of the p53-family, has a pivotal role in epithelial development. Knockdown of p63 expression resulted in the down-regulation of cell adhesion-associated genes such as integrin β4, β1, α6, fibronectin and laminin γ2 and caused cell detachment.21 Transfection of snail induced downregulation of p63 protein in SCC cells by inhibiting p63 promoter activity.22

Degradation of the ECM is involved in the process of cell detachment from the substratum and cell migration. Thus upregulation of the matrix metalloproteinases MMP-9 and MMP-2 in snail expressing cells has been observed.23-26 Furthermore, snail-expressing HepG2 cells enhanced the expression of MMP-1, MMP-2, MMP-7 and MT1-MMP.26 Although we also observed MMP-3 induction in snail expressing cells, neither the MMP-3 inhibitor nor MMP inhibitors efficiently suppressed cell detachment. In malignant mesothelioma cells, snail protein expression showed a positive association with MT1-MMP and TIMP-2 mRNA expression, but was unrelated to MMP-2 and MMP-9 expression or activity.27 Since the ECM is significantly changed in snail expressing cells, the sensitivity of these ECM proteins to proteases might be altered.

The Role of Snail in Apoptosis

There are several reports that implicate snail in cell survival. During embryonic development, expression of the snail gene in chicken and mouse is inversely correlated with cell death in different developing tissues.28 Snail downregulation by antisense oligonucleotides has been shown to increase cell death in colon tumors in a mouse model.29 Snail also confers resistance to cell death induced by the withdrawal of survival factors and by pro-apoptotic signals.28 The MAPK and PI3K survival pathways are highly active in snail expressing cells.28 Furthermore, snail expression also enhanced resistance to cell death elicited by DNA damage. A detailed molecular analysis of this phenomenon revealed that snail directly repressed the transcriptional of multiple factors that have well-documented roles in programmed cell death such as p53, BID and caspase-6.30 Anoikis refers to apoptosis induced by a loss of cell-matrix interactions. For a tumor cell to metastasize to a distant site, it needs to overcome anoikis.31 Metastatic dissemination generally occurs when cancer cells overcome anoikis after detachment from the primary tumor site. Although the transcription factor slug, that is another inducer of EMT, was reported to be essential for resistance to anoikis of human breast cells,31 snail did not confer resistance to anoikis.5 Loss of E-cadherin from cell-cell contacts is involved in the onset of anoikis.32 Given that snail represses the expression of E-cadherin, snail-expressing cells would fail to be resistant to anoikis.

The Role of Snail in Cell Attachment to ECM

To complete metastasis, tumor cells must adhere to some extracellular matrix ligands for migration and for reattachment to the second site. Snail expressing cells have enhanced expression of integrin αv or α5 and expression of αvβ3 integrin stimulates tumor cell adhesion to vitronectin.33 Thus it is possible that snail-expressing cells might show enhanced reattachment to ECM that contains fibronectin or vitronectin. Indeed, we did observe enhanced attachment of snail-expressing MDCK and A431 cells to tissue culture wells coated with fibronectin or fetal calf serum which could be detected as early as 30 min after plating. Decreased cell adhesion to laminin-332,34 within 72 hr after plating has been reported in slug-expressing epidermal keratinocytes. The production of ECM proteins and their corresponding

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Table 1  Adhesion-related genes differentially expressed in snail-expressing cells

| Genes downregulated in snail-expressing cells | Genes upregulated in snail-expressing cells |
|-----------------------------------------------|--------------------------------------------|
| Integrins                                        | Integrins                                    |
| α5, α6, β4                                      | α5                                          |
| α3, α5, β3, γ2                                  | αa1                                         |
| Aggrecan,59                                      | Fibronectin71                                |
| Type II collagen,59                             | MMP-1,26                                    |
| Type IV collagen,5                             | MMP-2,24,26                                 |
| Type X collagen,50                             | MMP-3,7,26                                  |
| MMP-9,23, MT1-MMP26                            | PAI-1,20                                    |
| Other extracellular matrix molecules            |                                             |
| p63,22                                         |                                             |
| Matrix metalloproteinases                        |                                             |
| Other detachment-related genes                   |                                             |
receptors in snail-expressing tumor cells is changed from the base-
ment membrane type, e.g., laminin332, to the stromal type, e.g.,
fibronectin. Rapid cell attachment to specific ECM ligands would
prevent anoikis, induce migration and enhance the re-growth of
metastasized snail-expressing tumor cells. Therefore blocking of
integrin-cell attachment might provide a therapeutic benefit for
the treatment of snail-expressing tumors.

The Role of Snail in Cell Migration

The snail genes are implicated in biological processes that
involve cell movement during embryonic development such as
migration of the neural crest of Xenopus35 and of the axial
mesendoderm of zebrafish.36 Snail also triggers migration of hepa-
toma HepG2,37 and oesophageal squamous cell carcinoma cells.38
Snail silencing dramatically reduced the ability of breast carcinoma
MDA-MB231 cells to migrate into collagen IV.2 Snail accelerates
expression.40,41 VEGF and the VEGF receptor
transforming growth factor beta (TGFβ) and bone morphogenic proteins (BMPs) are also
inducible through hypoxia.50 Gli mediate constitutive Hedgehog signaling in the
common skin cancer, basal cell carcinoma. Snail is rapidly induced
by Gli1.51 Notch directly upregulates snail expression in two ways:
first by binding of the Notch intracellular domain to the snail-1
promoter and second by Notch potentiation of hypoxia-inducible
factor 1alpha (HIF-1α) recruitment to the lysyl oxidase (LOX)
promoter and elevation of the hypoxia-induced upregulation of
LOX, which stabilizes the snail-1 protein.52 An human snail
promoter that contains the site of initiation of transcription has
been characterized.53 This promoter was activated in response
to addition of the phorbol ester PMA or to overexpression of
integrin-linked kinase (ILK) or oncogenes such as Ha-ras or v-Akt.
Although other regions of the promoter were required for complete
stimulation by Akt or ILK, a minimal fragment (-78/+59) was
sufficient to maintain mesenchymal specificity. Activity of this
minimal promoter and snail RNA levels were dependent on the
ERK signaling pathway. NFkappaB/p65 also stimulates snail
transcription through a region located immediately upstream of
the minimal promoter, between -194 and -78.53 The endothelin
Receptor (ET(A)R)/endothelin-1 (ET-1) autocrine pathway
increases the level of snail. Activation of ET(A)R by ET-1 triggers
an ILK-mediated signaling pathway leading to GSK-3β inhibition
and snail stabilization.54 Overexpression of ILK stimulates snail
expression and inhibition of ILK resulted in the inhibition of snail
gene transcription.55 Ultraviolet radiation (UVR), which activates
MAPK cascades, also stimulates snail expression in epidermal ker-
atinocytes. This induction was mediated, at least in part, through the
ERK and p38 MAPK cascades.56 Reactive oxygen species (ROS)
stimulate the expression of snail.57 Exposure of mouse
mammary epithelial cells to MMP-3 induces the expression of
Rac1, which causes an increase in ROS and expression of snail.57
Snail mRNA expression was increased under hypoxic condi-
tions in ovarian cancer cell lines.58 Hypoxia is known to induce
hypoxia-inducible factor-alpha (HIF-1α), which binds to hypoxia-
responsive elements of target genes and activates the transcription
of these genes. HIF-1α has been proposed to activate snail via
HIF-1α engagement of the hypoxia-responsive element found in
the snail promoter at position -86 to -82.59 The product of the
von Hippel-Lindau gene (VHL) ubiquitylates HIF-1α leading to
oxygen-dependent HIF-1α destruction. Therefore, reintroduc-
tion of wild-type VHL into CC-RCC [VHL(-/-)] cells markedly
reduced the expression of snail.59 Signaling of the estrogen receptor
negatively regulates snail expression.60 The product of human
MTA3 (metastasis-associated gene) is an estrogen-dependent
component of the Mi-2/NuRD transcriptional corepressor and
constitutes a key component of an estrogen-dependent pathway.
The absence of estrogen receptor or of MTA3 leads to aberrant
expression of snail.60,61 Recent studies have shown that snail binds
to its own promoter and represses its activity. These results indicate
the existence of a feed-back mechanism of regulation of snail
transcription. Although, the expression of snail can be induced by different pathways that act at the transcriptional level, a non-transcriptional mechanism that regulates snail activity has been described. Snail is highly unstable, with a short half-life of about 25 min. GSK-3 β binds to and phosphorylates snail at consensus motifs and regulates ubiquitylation of snail by β-Trcp. A variant of snail (snail-6SA), which cannot be phosphorylated at these sites, is much more stable. In agreement with these findings, Wnt signaling inhibits snail phosphorylation and consequently increases snail protein levels. The lysine residues at position 98 and 137 of snail are essential for snail stability, its functional cooperation with LOXL2/3 and for snail induction of EMT. LOXL2 appears to attenuate GSK3-β-dependent snail degradation. Oxidation of snail K98 and/or K137 by LOXL2 generates an intramolecular linkage in snail thereby inducing a conformational change which would mask GSK-3-β-dependent regulatory motifs. Blockage of the GSK-3 β phosphorylation sites leads to a more stable and a nuclear-localized snail protein. Snail function is controlled by its intracellular location. The cytosolic distribution of snail depends on its nuclear export by a CRM1-dependent mechanism, and a nuclear export sequence (NES) has been located in the regulatory domain of snail. Export of snail is controlled by phosphorylation of a Ser-rich sequence adjacent to this NES. In contrast, phosphorylation of snail on Ser(246) by p21-activating kinase 1 (p21AK) promotes snail’s accumulation in the nucleus as well as its repressor functions. On the other hand, GSK-3 β phosphorylates the NES of snail and induces its export to the cytoplasm. Importantly, the phosphorylation and subcellular distribution of snail are also controlled by cell attachment to the extracellular matrix. Suspended cells show higher levels of phosphorylated snail and an augmented snail extranuclear localization compared to cells attached to culture plates. These findings show the existence of an effective and finely tuned nontranscriptional mechanism of regulation of snail activity that is dependent on the extracellular environment.

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