WNT-DEPENDENT REGULATION OF THE E-CADHERIN REPRESSOR SNAIL
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Running Title: Wnt/GSK3β-TrCP-dependent regulation of Snail
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Down-regulation of E-cadherin marks the initiation of the epithelial-mesenchymal transition, a process exploited by invasive cancer cells. The zinc finger transcription factor, Snail, functions as a potent repressor of E-cadherin expression that can, acting alone or in concert with the Wnt/β-catenin/T cell factor axis, induce an epithelial-mesenchymal transition. While mechanisms that coordinate signaling events initiated by Snail and Wnt remain undefined, we demonstrate that Snail displays β-catenin-like canonical motifs that support its GSK3β-dependent phosphorylation, β-TrCP-directed ubiquitination and proteasomal degradation. Accordingly, Wnt signaling inhibits Snail phosphorylation and consequently increases Snail protein levels and activity, while driving an in vivo epithelial-mesenchymal transition that is suppressed following Snail knockdown. These findings define a potential mechanism whereby Wnt signaling stabilizes Snail and β-catenin proteins in tandem fashion so as to cooperatively engage transcriptional programs that control an epithelial-mesenchymal transition.

Introduction
E-cadherin, the prototypic member of the cadherin single-pass transmembrane glycoprotein family, regulates cell adhesion in epithelial cells in a Ca2+-dependent manner via homotypic interactions with E-cadherin molecules on opposing cell surfaces (1). The adhesive function of E-cadherin is dependent on its binding to the cytoplasmic α- and β-catenin proteins which serve to tether the cadherin to the actin cytoskeleton (1). During embryonic development, down-regulation of E-cadherin function marks the onset of a complex program wherein epithelial cells adopt a fibroblast-like phenotype and display tissue-invasive activity, a process termed the epithelial-mesenchymal transition (EMT) (1,2). Likewise, E-cadherin repression is thought to play a major role in the abnormal manifestation of EMT in epithelial-derived cancer types (3-5). In both development and cancer, the zinc-finger transcription factor, Snail, has been implicated in E-cadherin repression via its binding to elements in the E-cadherin promoter (6). Indeed, during development, Snail plays a required role in driving the EMT programs that mark gastrulation as well as neural crest development (1,3-6). In a similar, but misdirected fashion, neoplastic cells co-opt Snail function in order to adopt a mesenchymal cell-like invasive phenotype that characterizes their aberrant behavior (1,3-7).

While Snail plays a critical role in both physiologic and pathologic EMT (5-8), E-cadherin repression frequently occurs in tandem with activation of the Wnt signaling cascade (9-14). Of note, independent of its well-defined role in E-cadherin-dependent adhesion, β-catenin also participates in Wnt signaling (1,2). In the absence of a Wnt signal, cytosolic β-catenin is normally phosphorylated by glycogen synthase kinase 3β (GSK3β) at one or more serine or threonine residues in its amino (N)-terminal domain (1,2). The N-terminally phosphorylated β-catenin is then recognized and ubiquitinated by a multiprotein complex containing the F-box protein β-TrCP, with resultant degradation of the poly-ubiquitinated β-catenin in the proteasome (1,2). Alternatively, a subset of the 19 known Wnt genes found in mammals (e.g., Wnt-1) can activate a pathway that inhibits the ability of GSK3β to phosphorylate target substrates via a process dependent on protein disheveled (Dvl), with resultant increases in β-catenin levels (1,2). The stabilization of β-catenin consequently leads to both enhanced nuclear accumulation and enhanced
binding to members of the T cell factor (TCF) family of transcription factors (1,2). In turn, β-catenin/TCF complexes regulate the expression of a panoply of gene products that direct cell fate, polarity and proliferation (1,2).

Though Wnt signaling could conceivably stabilize the pool of cytosolic β-catenin that is released from E-cadherin-bound sites as a consequence of Snail-mediated E-cadherin repression (1,2,9), direct interplay between the Wnt and Snail systems has remained a subject of conjecture. Herein, we demonstrate that the Snail transcript encodes a series of β-catenin-like canonical motifs that support its GSK3-dependent phosphorylation, β-TrCP-directed ubiquitination and proteasomal degradation via a Wnt-1-regulatable process. As increasing evidence indicates that Wnt signals can impact on multiple cell functions in neoplastic tissues (e.g., 12-16), these findings support a model wherein activation of the Wnt-GSK3β signaling cascade regulates carcinoma cell phenotype by controlling β-catenin/TCF- and Snail-dependent transcriptional programs in tandem fashion.

**Experimental Procedures**

**Plasmids** - A human Snail cDNA with a carboxyl (C)-terminal FLAG (Flg) epitope tag was subcloned into three expression vectors: pCR3.1, to generate pCR3.1-Snail-Flg; a bicistronic pCMS-EGFP, to generate pCMS-EGFP-Snail-Flg; and the retroviral vector pBabePuro (5). A human Snail cDNA with both a hexa-histidine (6XHis) and Flg C-Terminal epitope tag was cloned into the vector pET21 to generate pET21-Snail-His for expression of recombinant Snail protein in E. coli. Snail mutant proteins with Flg epitope tails, including the S100A, S104A, S107A, S100,104A, S104,107A, S100,107A and S96A mutants, were constructed by PCR-based methods using a wild-type Snail cDNA as a template, followed by subcloning into the pCR3.1 vector. 6XHis-/Flg-tagged Snail mutants (i.e., S100,104A and Δ81-109) were also generated by PCR-based methods for expression in bacteria using the pET21 construct. The E-cadherin reporter gene constructs Ecad(-108)-Luc and Ecad(-108)/EboxA.MUT/EboxB.MUT/EboxC.MUT-Luc were described previously (5). A β-TrCP cDNA was obtained by RT-PCR-based methods, using total mRNA of 293 cells, and the β-TrCP cDNA subcloned into pCR3.1. The dominant negative mouse b-TrCP vector (dN-b-TrCP-myc) was kindly provided by Dr. ZJ Chen (UT-Southwestern, Dallas) (44). A pCR3.1 expression vector encoding HA-tagged ubiquitin was generated by RT-PCR-based methods using HeLa total RNA. HA-tagged Wnt-1 retroviral expression vector (pLNCX-mWnt-1-HA) was a gift from O. MacDougald (U of Michigan).

To generate the pSUPER-Snail-shRNA, annealed oligos 5'-GATCCAGGCC TTCAACTGCAAATAGTGTGCTGTCCTA TTTGCAGTTGAGGCCTTTTTTTTGAA-3' AND 5'-AGCTTTTCCAAAAAAAGGCC TTCAACTGCAAATAGGACAGCACACTA TTTGCAGTTGAGGCCTG-3' were inserted into pSUPER-retro vector (OligoEngine). The 19 nt human Snail target sequence (indicated in capital letters) was designed and verified to be specific for Snail by Blast search against the human genome, and RT-PCR, respectively. A control shRNA oligo was used to generate pSUPER-control.

**Cell Culture and Retroviral Gene Expression** - The MCF-7 and 293 cell lines was cultured in Dulbecco's modified Eagle's medium supplemented with 1 mM sodium pyruvate, 100 units/ml penicillin, 100 µg/ml streptomycin, and 10% calf serum. pBabePuro-Snail-Flg or pLNCX-mWnt-1-HA were used to generate retroviral stocks in 293 cells for subsequent infection of MCF-7 cells. Stable transfectants, MCF-7-Snail-Flg and MCF-7-Wnt-1-HA, were obtained from pooled clones selected with 600 µg/ml Puromycin or 400 µg/ml Geneticin (Invitrogen Life Technologies, Chicago, Il).

**Recombinant 6x His fusion protein and in vitro phosphorylation** - Recombinant His- and Flg-double-tagged Snail protein was expressed in pET21- transformed BL21 E. coli and purified by Ni-Ti agarose and imidazole elution, according to the manufacturer’s protocol (Pierce Biotechnology, Rockford, IL). Two µg of protein was used for in vitro phosphorylation with 500 µCi/µmol 32P-γ-ATP (ICN), and 75 U of
recombinant GSK3 (New England Biolabs, Inc., Beverly, MA) for 2 h at 25°C C.

**Antibodies, immunoprecipitation and immunoblot analysis** - Snail-Flg proteins were detected by anti-FLAG-M2 antibody (Sigma, St. Louis, MO), Snail-His/Flg proteins were detected with either anti-6XHis or anti-FLAG antibody (BD Biosciences Clontech, Palo Alto, CA), and phosphorylated Snail proteins were detected with anti-phosphoserine antibody (Upstate USA, Inc., Charlotte, NC). beta-TRCP (SC-15354) and GSK3 (4G-1E) antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and Upstate USA, Inc., respectively. Anti-chicken type IV collagen antibody II B12 was provided by T. Linsenmayer (Tufts University). For immunoprecipitation, whole cell Triton X-100 lysates were incubated with FLAG-M2 agarose (Sigma), and washed with lysis buffer. Proteins were resolved by 10% SDS-PAGE, transferred to nitrocellulose and bound antibodies visualized with HRP-conjugated secondary antibodies and the enhanced chemiluminescence (ECL) system (Pierce Biotechnology) (41). For detecting endogenous Snail, beta-catenin and E-cadherin, nuclear and cytoplasmic fractions (prepared according to the manufacturer’s protocol; NucBuster Protein Extraction Kit, Novagen) were prepared from MCF-7 cells. Following SDS-PAGE, proteins were detected with either an anti-Snail polyclonal antisera (5), anti-beta-catenin (BD Bioscience), anti-E-cadherin (Zymed) or anti-beta-actin (BD Bioscience).

**Pulse-chase analysis** - MCF-7 cells or 293 cells were transiently transfected with 1.0 µg of pCR3.1-Snail-Flg (FuGene-6; Roche Diagnostics, Indianapolis, IN). After 12 h, the media was changed, and the cells were incubated in standard media for an additional 48 h, at which point, the cells were pulse-labeled with 50 µCi/ml of [35S]-Met/Cys (Perkin Elmer, Boston, MA) for 20 min, washed and incubated in complete media for indicated times. Cell lysates were immunoprecipitated with anti-FLAG-M2 agarose beads and the samples were subjected to SDS-PAGE and autoradiography.

**siRNA and shRNA targeting** - 293 cells were electrophorated with 100 nM of siRNA duplexes directed against human GSK3β or a scrambled mock siRNA (Upstate) 24 h after being transfected with 1.0 µg of pCMS-EGFP-Snail-Flg. Cells were harvested 48 h later, and GSK3 and Snail protein levels determined by immunoblotting with anti-GSK3 and anti-FLAG-M2 antibodies. MCF-7-Wnt-1 cells were transiently co-transfected with 1 µg of pSUPER-Snail-shRNA or pSUPER-control, and 0.5 µg of a pCMS-EGFP plasmid (BD Biosciences Clontech). Cells were collected 24 h after transfection.

**In vitro translation and in vitro binding assay** - [35S]-beta-TrCP protein was obtained by in vitro transcription and translation from the pCR3.1-beta-TrCP vector, using the TNT in vitro translation system (Promega, Madison, WI) with 10 μCi of [35S]-Met/Cys (Amersham, Piscataway, NJ), according to the manufacturer’s instruction. Lysates from 293 cells transiently transfected with wild-type or mutant Snail were immunoprecipitated with FLAG-agarose beads and equivalent amounts of the Snail proteins (normalized by semi-quantitative immunoblot analysis) incubated with in vitro translated [35S]-beta-TrCP for an additional 2 h at 25°C C. Specifically bound proteins were eluted, resolved by SDS-PAGE and visualized by immunoblotting.

**In vivo ubiquitination** - HA-tagged ubiquitin was co-transfected with each FLAG-tagged Snail construct. After 48 h, lysates were immunoprecipitated and immunoblotted with alpha-Flg or anti-HA antibodies.

**Wnt treatment** - Cell and extracellular protein extracts from Wnt-1 expressing RAC-311 and control MV7 cells (both cell lines kindly provided by L.R. Howe) were prepared from a 0.4 M NaCl buffer extract as described previously (45), and added to cells at a final concentration of 30 µg/ml.

**Reporter gene assays** - MCF-7 cells were co-transfected with 1.0 µg of reporter gene Ecad(-108)-Luc or Ecad(-108)/EboxA.MUT/EboxB.MUT (5). Ecad(-108), Ecad(-108)/EboxA.MUT/EboxB.MUT, 25 ng of wild-type or mutant Snail expression vectors; MCF-7-Snail-Flg or MCF-7-Wnt-1-HA cells were transfected with 1.0 µg of reporter gene only (5). Reporter gene activities were measured with Luciferase Assay System (Promega) 48 hrs after transfection and normalized by measuring beta-gal activities of co-transfected pSV-gal (0.25 µg, Clontech) with beta-Galactosidase Assay System (Promega). Reporter gene activities were presented as relative light units (RLU) to that reference.
obtained from mock-transfected cells. Results are expressed as the average of the ratio of the reporter activities.

**Chick Chorioallantoic Membrane Invasion Assay** - Control transfected MCF-7 cells, wild-type Snail- or mutant Snail- transfected cells labeled with fluoresbrite carboxylate microspheres (Polysciences) were cultured atop the CAM of 11-day-old chick embryos for 3 d (25). For analyses of MCF-7-Wnt-1 expressing cells, invasion of the GFP/siRNA co-transfected cells was monitored by GFP fluorescence in place of the fluorescent beads.

**Immunofluorescence** - MCF-7 cells fixed with 4% formaldehyde, permeabilized with 1% Triton-100 and immunostained with anti-E-cadherin antibody and Alexa-Fluoro-488 labeled anti-mouse secondary antibody (Molecular Probe, Eugene, OR). FLAG-tagged Snail was stained with anti-FLAG polyclonal antibody (Sigma, Eugene, OR). FLAG-tagged Snail was stained with anti-FLAG polyclonal antibody (Sigma, 1:500) and Alexa-Fluoro-594 labeled anti-rabbit secondary antibody (Molecular Probe).

**Results**

Following retroviral transduction of MCF-7 cells with a vector encoding a FLAG epitope-tagged form of human Snail (Snail-Flg), the cells displayed a morphology consistent with that seen in a typical EMT, and E-cadherin protein expression was strongly suppressed (Fig. 1A, B). To determine if Snail can trigger tissue-invasive activity in a manner consistent with the acquisition of a mesenchymal cell-like phenotype, fluorescently-labeled MCF-7 cells were cultured atop the chick chorioallantoic membrane. Whereas mock-transduced MCF-7 cells were confined to the upper surface of the CAM, the Snail-expressing MCF-7 cells perforated the underlying basement membrane and invaded into the CAM interstitium (Fig. 1A). Interestingly, when the Snail protein was recovered from transfected MCF-7 (or 293) cells, it migrated as a closely spaced doublet, suggesting post-translational modifications (Fig. 1C). While treatment with O- or N-glycanase failed to alter the migration pattern of Snail (data not shown), treatment of protein extracts with λ protein phosphatase (λPPase) resulted in the loss of the more slowly migrating Snail isoform (Fig. 1C), consistent with notion that Snail is phosphorylated in MCF-7 as well as 293 cells (Fig. 1C). The human Snail amino acid sequence contains in excess of 20 serine and/or threonine residues, but the search for Snail domains that might be phosphorylated was considerably narrowed as preliminary studies indicated that a mutant form of Snail lacking amino acids 92-120 migrated as a single band (data not shown). Of note, inspection of this region of the Snail protein revealed a S92(X)2DSG(X)2S(X)3S(X)2S107 motif with considerable similarity to the GSK3β-sensitive N-terminal phosphorylation motif found in β-catenin (Fig. 1D) (1,2). Further, this region of the Snail protein also contained the DSGXXS destruction motif recognized by β-TrCP in β-catenin as well as the IκB protein (Fig. 1D) (1,2).

Consistent with the possibility that Snail, like β-catenin, is recognized and bound by GSK3β (1,2), immunoprecipitates of Snail-Flg recovered from transfected 293 cells contained endogenous GSK3β (Fig. 2A). In light of the existence of multiple potential phosphorylation sites within the amino acid 92-120 region of Snail, a series of single, or double, Ser→Ala substitutions were generated in the Snail-Flg expression vector. The constructs encoding the Snail mutants were then expressed in 293 cells, and the electrophoretic mobility of the Snail-Flg proteins was examined prior to and following λPPase treatment of the cell extracts. Though single Ser→Ala substitutions at Ser100 (S100A), Ser104 (S104A) or Ser107 (S107A), or double substitutions at Ser100/Ser104 (S100,104A) or Ser100/Ser107 (S100,107A) did not affect the λPPase-induced gel shift, dual Ser→Ala mutations at Ser104 and Ser107 (S104,107A) ablated the gel shift associated with λPPase treatment (Fig. 2B). A key role for the Ser104 and Ser107 residues was further corroborated by data showing that recombinant GSK3β directly phosphorylated wild-type Snail via a LiCl-sensitive process in vitro (17), but not the S104,107A double mutant form of Snail (Fig. 2C). Indeed, whereas pulse-chase analysis demonstrated wild-type Snail was rapidly degraded in the course of a 4 h chase period, expression levels of the S104,107A mutant remained stable (Fig. 2D).

The inability of GSK3β to phosphorylate the S104,107A mutant Snail protein in vivo could conceivably reflect non-specific alterations in the conformation of the mutant Snail (18). However, consistent with the notion that Snail is
phosphorylated in vivo by GSK3β, phosphorylation of wild-type Snail in 293 cells was blocked by treatment of the cells with the GSK3β inhibitor, LiCl (Fig. 2E) (17,18). Coincident with the LiCl-dependent inhibition of Snail phosphorylation, steady state levels of wild-type Snail were also increased in LiCl-treated cells (Fig. 2E). Additionally, pulse chase data indicated that LiCl treatment prolongs the half-life of the faster migrating and presumably, hypo- or non-phosphorylated form(s) of Snail (Fig. 2E). Using a siRNA knockdown approach to reduce endogenous GSK3β levels in 293 cells engineered to ectopically express Snail, the steady state levels of Snail protein were likewise increased (Fig. 2F).

To further determine whether GSK3β regulates the endogenous level of Snail in a fashion similar to that observed for the exogenously expressed protein, MCF-7 cells were cultured in the presence of either LiCl or the GSK3-specific inhibitor, CHIR99021 (19). As expected, GSK3 inhibition by either reagent induced significant increases in the steady state nuclear levels of endogenous Snail in tandem with similar increases in β-catenin (Fig. 2G).

In vivo, a subset of Wnt family members trigger largely undefined pathways that lead to the inhibition of GSK3β activity (1,20). As Wnt expression occurs frequently in a temporal fashion coincident with developmental EMT programs (1,20-22), we sought to determine whether GSK3β-dependent phosphorylation of Snail could be inhibited by Wnt-1. Indeed, the phosphorylation of ectopically expressed wild-type Snail was suppressed markedly by treatment of the cells with Wnt-1-conditioned media, and in concert, Snail half-life was increased (Fig. 3A, B). While Wnt-1 also increased the steady state expression levels of wild-type Snail, no effect was observed on the levels of the S104,107A mutant (Fig. 3C). Consistent with the premise that GSK3β regulates Snail and β-catenin levels in a cooperative fashion, Wnt-1 signaling mediated tandem increases in the nuclear levels of endogenous Snail as well as β-catenin (Fig. 3D).

Following the GSK3β-dependent phosphorylation of β-catenin, a D55SpGXXSp motif in the post-translationally modified protein is recognized by the E3 ubiquitin ligases, β-TrCP1 or β-TrCP2, which mediate the ubiquitination of phosphorylated β-catenin and target the protein for subsequent degradation in the proteasome (1,2,23). To determine whether β-TrCP recognized the homologous D55SGXXS motif found in Snail, a FLAG epitope-tagged wild-type Snail protein was ectopically expressed in 293 cells. Following immunoprecipitation of the extracts with an anti-FLAG antibody, endogenous β-TrCP1 protein was found to be specifically recovered in association with Snail (Fig. 4A). Furthermore, [35S-Met]-labeled β-TrCP1 prepared by in vitro translation, was shown to bind to the wild-type Snail protein recovered from lysates of transfected 293 cells (Fig. 4B). However, β-TrCP1 was unable to form a stable complex with either the phosphorylation-resistant Ser104,107A mutant Snail protein or the Snail mutant harboring a single Ser96→Ala substitution within the putative β-TrCP1-binding domain (Fig. 4B). Consistent with these findings, the steady-state levels of wild-type Snail were enhanced by co-expressing a dominant-negative form β-TrCP1 in 293 cells (Fig. 4C) as well as MCF-7 cells (data not shown).

In a fashion similar to that described for β-catenin (1,2,22), further studies demonstrated that wild-type Snail protein undergoes rapid ubiquitination in vivo (Fig. 4D). By contrast, ubiquitination of Snail proteins containing mutations at either key phosphorylation or recognition sites (i.e., Ser104/Ser107 or the Ser96 within the DSG destruction motif), was strongly suppressed, as was the formation of high molecular weight Snail complexes in Wnt-1-treated cells (Fig. 4D, E). While the Ser96→Ala mutant Snail protein was phosphorylated in 293 cells, the loss of this key phosphorylation site within the DSG destruction motif resulted in the failure of the protein to be recognized and ubiquitinated by β-TrCP, resulting in an increased steady state level and prolonged half-life of the mutant (Fig. 4B,D,F). Indeed, the half-life of the Ser96→Ala mutant Snail protein was similar to that observed following treatment of 293 cells expressing wild-type Snail protein with the proteasome inhibitors, lactacystin or MG-132 (Fig. 4G).

Snail has been proposed to play a key role during both development and cancer by virtue of its ability to repress E-cadherin expression and drive EMT (3-5). To assess the consequences of
Snail stabilization on its E-cadherin repressor activity, we compared the relative abilities of wild-type Snail and the Ser104,107A or Ser96→Ala mutants to regulate an E-cadherin promoter gene construct previously shown to be Snail-sensitive (3-5). Because Snail has been implicated in repression of E-cadherin transcription through the three E-box domains present in the proximal E-cadherin promoter region, a control version of the construct was employed wherein all three E-box domains were mutated (3X Mut) (5). Whereas limiting amounts (25 ng) of the expression vector encoding wild-type Snail exerted only modest inhibitory effects on the wild-type E-cadherin reporter gene construct in MCF-7 cells, equivalent amounts of the expression vectors encoding the Ser104/Ser107 or Ser 96→Ala mutants exerted stronger repression effects on E-cadherin promoter activity (Fig. 5A). An analysis of the levels of Snail transcripts expressed ectopically in the cells confirmed that the differences in repression activity observed between the wild-type and mutant Snail constructs did not result from variations in the mass of DNA-transfected, or the levels of transcripts produced from the vectors (Fig. 5A). When compared to wild-type Snail, the Ser104/Ser107 and Ser96→Ala mutants also displayed an enhanced activity to suppress endogenous E-cadherin expression in transfected MCF-7 cells (Fig. 5B). Though phosphorylation of Snail has been reported to regulate its nuclear localization and repressor activity by modulating the activity of a nuclear export sequence located between residues 132 and 143 (24), both the Ser104/Ser107 and the Ser96→Ala mutants were preferentially localized to the nuclear compartment in a fashion comparable to wild-type Snail following transfection into MCF-7 cells (Fig. 5B).

To address the possibility that stabilized Snail might play an exaggerated role in engaging an EMT program capable of promoting an invasive phenotype, MCF-7 cells were transiently transfected with wild-type Snail or either of the Ser104,107A or Ser96→Ala mutants, and the in vivo invasive behavior of the MCF-7 transfectants assessed by culturing the cells atop the chick CAM. While control-transfected MCF-7 cells were unable to penetrate the CAM surface, both the S104,107A and the Ser96→Ala mutants induced a more aggressive tissue-invasive program than wild-type Snail (Fig. 5C).

Finally, given the ability of Wnt-1 to induce the up-regulation of endogenous levels of nuclear Snail protein, coupled with its ability to repress E-cadherin expression and support a tissue-invasive EMT program, we sought to determine whether Wnt-1 drives EMT via a Snail-dependent process. Indeed, following Wnt-1-transduction, MCF-7 cells adopted a fibroblastic phenotype, down-regulated E-cadherin levels, increased nuclear concentrations of β-catenin and Snail proteins, and suppressed E-cadherin promoter activity (Fig. 6A, B and C). Moreover, consistent with the proposition that a Wnt-driven EMT program has been induced, the Wnt-1-transduced MCF-7 cells expressed tissue-invasive activity similar to that observed with Snail-transduced cells (compare Fig. 6C with Fig. 1A). If, however, Snail expression in Wnt-transduced MCF-7 cells was targeted with any one of four different shRNA constructs, Snail mRNA expression was inhibited completely (data not shown), the suppression of E-cadherin promoter activity was reversed (Fig. 6B), and tissue-invasive activity was lost (Fig. 6C). By contrast, transfection with a control shRNA neither affected E-cadherin promoter activity or invasion (Fig. 6B and C). Hence, Snail plays a key role in regulating Wnt-1-induced EMT in MCF-7 cells.

**Discussion**

Snail plays a key role in EMT processes characterizing tumor progression, as well as developmental programs ranging from gastrulation to neural crest formation (6). The unexpected identification of a β-catenin-like consensus motif in the N-terminal domain of Snail that supports its GSK3β-dependent phosphorylation, β-TrCP-dependent ubiquitination and proteosomal degradation highlights a new model wherein Wnt signaling participates in the co-regulation of Snail- as well as β-catenin/LEF-1-driven transcriptional programs. With regard to β-catenin, its dual phosphorylation by GSK3β and casein kinase-1 (CK-1) appears to take place in a multi-protein complex that includes APC (adenomatous polyposis coli) and Axin tumor suppressor proteins (1,6,13). While a required role for CK-1 in generating a phosphoserine priming site in β-
in vitro phosphorylate Snail. Preliminary studies suggest that CK-1 can phosphorylate Snail in vitro (unpublished observation). The ability of GSK3β to phosphorylate primed versus unprimed Snail in vivo, however, requires further study. These findings notwithstanding, it is conceivable that Snail, like β-catenin, might be stabilized by mutations in APC, Axin or in the Snail transcript itself, thus predisposing to the development of carcinogenic states (3-5,29). However, as increasing evidence supports important roles for the activation of canonical Wnt signaling in normal as well as neoplastic states (e.g., 12-15,30,31), inappropriate activation of Snail-dependent transcriptional programs are not necessarily constrained to pathological processes that require somatic mutations in the Snail gene itself or accessory molecules that regulate its phosphorylation and degradation.

During embryogenesis, E-cadherin downregulation appears to be temporally linked to FGF signaling, Snail expression, and activation of the canonical Wnt signaling cascade (9-11,20,21,32). Recent studies suggest that β-catenin, upon its association with the TCF transcription factor family member, LEF-1, can act in a cooperative fashion with Snail to suppress E-cadherin transcription via LEF-1/β-catenin interactions with sequences upstream of the E-cadherin promoter (11). Our findings raise the possibility of a more complex and interdigitated scheme wherein exposure of normal or neoplastic cells to a combination of Snail-inducing growth factors and Wnts would stabilize the intracellular levels of the Snail and β-catenin proteins by as yet undefined mechanisms that serve to shield these regulatory molecules from GSK3β-dependent phosphorylation (1,20,33). In turn, Snail acting as a transcriptional repressor of E-cadherin, potentially facilitates the intracellular transfer of E-cadherin-bound β-catenin to a Wnt-stabilized “signaling” pool (3-5,9). Snail might further synergize with the Wnt/β-catenin axis by inducing LEF-1 expression either directly or by suppressing BMP expression (11,29,34). Additional studies are needed to determine the relative roles that stabilized Snail and β-catenin (and perhaps, γ-catenin; ref 35) play in coordinating Wnt-induced EMT programs, but Snail appears to play a dominant, if not required, role in driving the tissue-invasive process in MCF-7 breast carcinoma cells. Given the parallels noted between developmental and neoplastic EMT programs (6), these findings are consistent with a required role for Snail during formation of the mesoderm germ layer (36).

Interestingly, our results complement and extend a very recent report by Zhou et al which similarly describes a role for GSK3β in regulating Snail phosphorylation and activity (37). In a variation of the theme presented here, these investigators proposed that Snail displays two distinct GSK3β consensus motifs. The first GSK3β recognition sequence, as in our study, centers on the putative β-TrCP binding site at phospho-Ser96 and phospho-Ser107. They then propose that a second motif, Ser107XXXSer111XXXSer115XXXSer119, regulates the export of nuclear Snail into the cytosolic space as a consequence of an independent series of GSK3β-dependent phosphorylations (37). Further work is required to cohesively mesh this model with our independent findings that describe a dominant role for Ser104 and Ser107 in regulating Snail phosphorylation and activity. However, it should be noted that GSK3β effectively phosphorylates serine or threonine residues in vivo only when a phosphoserine or phosphothreonine is positioned four amino acids C-terminal to the new phosphorylation site (18). Hence, we tentatively favor a working model wherein a priming event first occurs at Ser104 which would simultaneously support the serial GSK3β-dependent phosphorylation of Ser104, Ser96 and possibly Ser92 as well as the CK-1-dependent phosphorylation of Ser107 (18). While mass spectrometric analyses will help resolve these issues, it should be emphasized that our findings further diverge from those of Zhou et al with regard to the functional ramifications of the GSK3β-Snail axis (37,38). In contrast with our report, these investigators reported that wild-type Snail – even when overexpressed in MCF-7 cells – is unable to induce EMT (37). Further, while Wnt signaling was not examined in their study, no agonists could be identified that were able to either induce increases in endogenous Snail or engage Snail-dependent EMT (37,38). Instead, EMT was only observed when a series of six Ser→Ala
substitutions were introduced into the Snail protein at Ser$^{96}$, Ser$^{100}$, Ser$^{107}$, Ser$^{111}$, Ser$^{115}$ and Ser$^{119}$, and the mutant construct overexpressed in target cells (37). Hence, our demonstration that Wnt signaling not only regulates endogenous Snail, but also drives Snail-dependent EMT, represents the first functional demonstration of the potential importance of this pathway in neoplastic cells.

By embedding within Snail a series of β-catenin-like recognition motifs for GSK3β and β-TrCP, a cooperative system appears to have evolved that allows for the tandem regulation of Snail- and β-catenin/TCF-regulated target genes as they converge on the EMT process. Of note, while the S$^{92}$(X)$_2$DSG(X)$_2$S(X)$_2$S(X)$_2$S$^{107}$ motif is conserved in human as well as other mammals (Fig. 1), substitutions are observed in more distinctly related vertebrate species (see Table I) where it has already been noted that Snail family members display highly divergent amino-terminal regions (6). While differences in Snail function among species make direct comparisons between conserved regions difficult to interpret [e.g., Slug, rather than Snail, plays the dominant role in driving EMT in the chick; (6)], the $^{96}$S→E substitution is consistent with more recent studies demonstrating the ability of β-TrCP to both recognize a charged glutamate residue in place of a phospho-Ser moiety (39) as well as target substrates harboring a DSG(X)$_n$S motif where n>2 (23). We do note, however, that the bulk of the putative nuclear localization signal assigned by Zhou et al to the Ser$^{111}$XXXSer$^{115}$XXXSer$^{119}$ motif (37) is not conserved in lower vertebrates (see Table I). This caveat aside, it appears that a common, but modified, scheme for regulating Snail activity may extend beyond mammals to include less closely related vertebrate species where constraints specific to given organisms have resulted in the evolution of a set of similar, yet distinct, molecular solutions.

Until recently, attention has focused on the role of mutational defects in APC, β-catenin, and Axin in activating the Wnt/β-catenin/TCF pathway in human cancer. Intriguingly, while mutational defects in β-catenin, Axin, and APC are fairly common in certain cancer types, in other neoplastic states such as breast cancer, mutational defects in well-defined components of the canonical Wnt pathway have only very rarely been identified (5,40). These findings could be interpreted as evidence Wnt signaling cascades play only a limited role in the development and progression of breast and other cancers where mutational defects in the canonical Wnt signaling pathway have rarely been seen. However, recent studies have suggested an important functional role for Wnt signals in various cancers and the possibility that non-mutational mechanisms may activate Wnt signaling via either enhanced local (autocrine or paracrine) expression of Wnts, epigenetic inactivation of the expression of Wnt antagonists (such as SFRPs or WIF-1), or the up-regulation of potential GSK3β inhibitor factors (12,14,16,40). Curiously, and inconsistent with the view that Wnt signals in cancer cells transmit their effects solely via stabilization of β-catenin, a requirement for upstream Wnt ligand function/activity has even been implicated in cancers with constitutive deregulation of β-catenin resulting from APC or β-catenin mutations (13-15). Taken together, these data support a model wherein inappropriate activation of the Wnt-GSK3β signaling cascade regulates carcinoma cell phenotype not only via effects on β-catenin/TCF-dependent transcription, but also through the ability of the Wnt-GSK3β signaling cascade to activate Snail-driven transcriptional programs. Indeed, given that Snail activity extends beyond the regulation of EMT-related processes to include cell death and growth (e.g., 8,41-43) our findings support a new operating platform by which canonical Wnt signals can regulate tumor cell phenotype.

References

Footnotes

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Figure Legends

Figure 1. The E-cadherin Repressor, Snail, induces EMT in MCF-7 cells and is target for phosphorylation. (A) MCF-7 cells, transduced with control (control, empty vector) or Snail-expressing retroviruses, were fixed and stained with an anti-E-cadherin antibody. Phase contrast (upper panels) and indirect immunofluorescence (lower panels) images are displayed (green, E-cadherin; blue, DAPI stained nuclei). In the lower panels, control- or Snail-expressing MCF-7 cells were labeled with fluorescent beads (green) and cultured atop the chick CAM for 3 d. CAMS were fixed, nuclei stained with DAPI (blue) and the CAM basement membrane stained with anti-chick type IV collagen antibody (red). Frozen sections were examined by fluorescence microscopy. The upper edge of the CAM surface is marked by the black arrows and the invasive tumor cells by white arrows. (B) E-cadherin protein level in total cell lysates from empty or Snail-transfected MCF-7 cells was assessed by immunoblot analysis with ß-actin serving as the loading control. (C) MCF-7 or 293 cells were transiently transfected with Snail-Flg, and metabolically labeled with [35S]-Met/Cys. Snail was immunoprecipitated from cell lysates with ß-Flg (ß-Flg)/agarose beads and subjected to SDS-Page followed by autoradiography (lanes labeled “[35S]”) or immunoblot analysis (IB). Following treatment of cell lysates recovered from Snail-Flg-transfected 293 cells with 200 U λ protein phosphatase (30 min @ 37° C), the Snail doublet detected by α-Flg collapsed to a single, faster migrating band. (D) Sequence alignment of ßB and ß-catenin with human (h), mouse (m), rat (r), pig (p) and dog (d) Snail. Highlights are shown for conserved serine residues (yellow) and the DSG motif (blue).

Figure 2. GSK3ß-Dependent Snail Phosphorylation. (A) GSK3 (both ß and ß) were detected in control- and Snail-Flg-transfected 293 cell lysates by immunoblot analysis (upper). GSK3ß-Snail complexes were isolated in immunoprecipitates recovered from lysates of control- or Snail-Flg-
transfected 293 cells following pull-down with α-Flag/agarose beads, SDS-PAGE and immunoblot with anti-GSK3 antibody (middle). Snail protein levels in the control- or Snail-Flag-transfected cells were assessed by α-Flag blot (lower). (B) Flag-tagged Snail mutants harboring Ser→Ala substitutions were expressed in 293 cells, lysates from the transfectants incubated with or without λ protein phosphatase, and changes in Snail mobility analyzed by α-Flag immunoblot. (C) His-tagged recombinant wild-type Snail, S104,107A mutant Snail, or a Δ81-109 Snail deletion mutant (2 µg protein each) were incubated with recombinant GSK3β and [32P]γ-ATP for 2 h at 25°C. Phosphorylated and total Snail protein were resolved by SDS-PAGE and visualized by autoradiography or α-Flag blot (upper). Snail phosphorylation by GSK3β was inhibited by LiCl (20 mM) (lower). (D) Wild-type or S104,107A mutant Snail-Flag was expressed in 293 cells, immunoprecipitated with α-Flag and immunoblotted with either anti-phosphoserine (anti-pSer) or α-Flag antibodies (upper). Following a 20 min pulse with [35S]-Met/Cys, 293 cells transfected with wild-type Snail-Flag or S104,107A Snail-Flag were lysed at the indicated times, α-Flag immunoprecipitates resolved by SDS-PAGE and visualized by autoradiography (lower panel). (E) Phosphorylated and total Snail protein levels were monitored in Snail-Flag transfected 293 cells incubated with or without 40 mM LiCl for 18 h (upper). Snail half-life in LiCl treated cells were measured by pulse-chase analysis (lower panel) as described above. (F) 293 cells were transiently transfected with an GFP-Flag-Snail expression vector and either GSK3-α/β and Snail levels were monitored by immunoprecipitation with α-GSK3 and α-Flag immunoblot. GFP, detected with α-GFP, was used as the loading control. (G) MCF-7 cells (1x10⁶) were incubated in the absence or presence of LiCl (40 mM) or CHIR99021 (2 µM) for 8 h. Following SDS-PAGE of nuclear extracts, endogenous Snail and β-catenin and actin were detected by immunoblot. Cytosolic β-actin was used as loading control.

**Figure 3. Wnt-1 Regulation of Snail Phosphorylation.** (A) 293 cells transfected with Snail-Flag were treated with control or Wnt-1 conditioned media for 18 h. Phosphorylated Snail levels were determined following immunoprecipitation (1x and 1/2x volume of the respective cell lysates) with α-Flag and immunoblotting with anti-pSer. (B) Snail half-life in control- or Wnt-1- treated 293 cells was determined by pulse-chase analysis and SDS-PAGE/autoradiography of α-Flag immunoprecipitates. (C) 293 cells were transfected with either GFP-Flag-Snail-Flg or S104,107A Snail-Flag and incubated with control- or Wnt-1-conditioned cell extracts for 18 h. Snail, GFP and β-actin were detected with α-Flag, α-GFP and α-β-actin immunoblot. (D) MCF-7 cells (1x10⁶) were incubated with control- or Wnt-1- conditioned cell extracts for 18 h. MCF-7 nuclear extracts were immunoblotted for endogenous Snail or β-catenin and actin were detected by immunoblot. Cytosolic β-actin was used as loading control.

**Figure 4. Phosphorylation-Dependent Snail Ubiquitination and Proteasome Degradation.** (A) Endogenous β-TrCP isoforms were detected in lysates of control- or Snail-Flag-transfected 293 cells (upper). β-TrCP1/Snail-Flag complexes were identified in α-Flag/agarose immunoprecipitates following immunoblotting with anti-β-TrCP (lower panel). (B) Equivalent amounts of wild-type Snail, S107,104A and S96→A Snail proteins were harvested from transfected 293 cells, immobilized on α-Flag-agarose beads, and incubated with in vitro-translated [35S]-β-TrCP1, bead-bound protein was resolved by SDS-PAGE and autoradiography (upper) or detected by α-Flag immunoblotting (lower). (C) 293 cells were transfected with a pCMS-EGFP-Snail-Flg alone or with dN-TrCP1-Myc for 48 h. Protein levels of dN-TrCP1 were detected with anti-Myc (upper), Snail with anti-FLAG (middle) and anti-GFP as control (lower) by Western Blot. (D) 293 cells were co-transfected with HA-tagged ubiquitin and wild-type, S104,107A or S96A Snail. Following immunoprecipitation with α-Flag, ubiquitinated Snail (upper) was visualized following SDS-PAGE and anti-HA immunoblotting. Snail monomers were detected by immunoprecipitating and immunoblotting with α-Flag (lower). (E) 293 cells expressing Snail-Flag were incubated with control- or Wnt-1- conditioned cell extracts for 18 h. Snail monomers and high molecular weight forms of Snail were detected by immunoblotting with α-Flag. (F) The phosphorylation (upper) and half-life (lower) of wild-type versus S96A Snail protein were monitored in 293 cells transfected with the respective FLAG-tagged Snail constructs. (G) 293 cells transfected with a wild-type Snail-Flag expression
vector were incubated with lactacystin (5 μM), MG-132 (5 μM) or DMSO for 18 h. Lysates from DMSO- or inhibitor-treated cells (1.0 μg and 0.2 μg total protein, respectively) were immunoblotted for Snail protein content (upper). Pulse-chase analysis of Snail-Flg half-life was likewise performed in proteasome inhibitor-treated cells (lower). Inhibitors were added to the transfected cells 1 h before the pulse and included during the entire chase period.

**Figure 5. Regulation of E-cadherin Repressor and Invasive Activities by Degradation-Resistant Snail Mutants.** (A) The E-cadherin repressor activity of wild-type, S104,107A and S96A Snail were assessed with the reporter construct, Ecad(-108)-Luc, which contains the wild-type promoter sequence from nt -108 to +125 of the endogenous E-cadherin promoter or a control construct, Ecad(-108)/EboxA.MUT/EboxB.MUT/EboxC.MUT-Luc, which harbors mutations in all three E-boxes (3xMut). Snail mRNA levels in each of the transfectants was monitored by RT-PCR. (B) E-cadherin expression in MCF-7 cells transiently transfected with wild-type S104,107A or S96A Snail was assessed by indirect immunofluorescence with anti-E-cadherin mAb (green). Snail was stained with α-Flag rabbit polyclonal antibody (red). Images were obtained by confocal microscopy. (C) MCF-7 cells were transiently transfected with control, wild-type, S104-107A and S96A Snail. CAMs were fixed, cell nuclei stained with DAPI (blue) and tissue sections examined by fluorescence microscopy. The upper edge of the CAM surfaces is marked by the black arrows. Numbers of invaded cells were counted in 10 randomly selected fields for each experiment and expressed as the mean ± 1 SD (Control: 0±0, Wild-type: 4±2, S104,107A: 30±6, and S96A: 48±4).

**Figure 6. Involvement of Snail in Wnt-induced Down-regulation of E-cadherin and Invasion.** (A) MCF-7 cells, transduced with control (control, empty vector) or WNT-1-expressing retroviruses, were incubated with lactacystin (5 μM), MG-132 (5 μM) or DMSO for 18 h. Lysates from DMSO- or inhibitor-treated cells (1.0 μg and 0.2 μg total protein, respectively) were immunoblotted for Snail protein content (upper). Pulse-chase analysis of Snail-Flg half-life was likewise performed in proteasome inhibitor-treated cells (lower). Inhibitors were added to the transfected cells 1 h before the pulse and included during the entire chase period.

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## Table 1. Snail Serine-Rich Region Sequence Alignment

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