Published in final edited form as:

Prostate. 2010 June 15; 70(9): 982–992. doi:10.1002/pros.21132.

Snail Transcription Factor Regulates Neuroendocrine Differentiation in LNCaP Prostate Cancer Cells

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Abstract

Background—Snail transcription factor induces epithelial-mesenchymal transition (EMT) via decreased cell adhesion-associated molecules like E-cadherin, and increased mesenchymal markers like vimentin. We previously established Snail-mediated EMT model utilizing androgen-dependent LNCaP cells. These cells express increased vimentin protein and relocalization of E-cadherin from the cell membrane to the cytosol. Interestingly, Snail transfection in LNCaP cells resulted in cells acquiring a neuroendocrine-like morphology with long neurite-like processes.

Methods—We tested for expression of neuroendocrine markers neuron specific enolase (NSE) and chromogranin A (CgA) by Western blot analysis, and performed proliferation assays to test for paracrine cell proliferation.

Results—LNCaP cells transfected with Snail displayed increase in the neuroendocrine markers, NSE and CgA as well as translocation of androgen receptor to the nucleus. LNCaP C-33 cells that have been previously published as a Neuroendocrine Differentiation (NED) model exhibited increased expression levels of Snail protein as compared to LNCaP parental cells. Functionally, conditioned medium from the LNCaP-Snail transfected cells increased proliferation of parental LNCaP and PC-3 cells, which could be abrogated by NSE/CgA siRNA. Additionally, NED in LNCaP-C33 cells or that induced in parental LNCaP cells by serum starvation could be inhibited by knockdown of Snail with siRNA.

Conclusion—Overall our data provide evidence that Snail transcription factor may promote tumor aggressiveness in the LNCaP cells through multiple processes; induction of EMT may be required to promote migration, while NED may promote tumor proliferation by a paracrine mechanism. Therefore, therapeutic targeting of Snail may prove beneficial in not only abrogating EMT but also NED.

Keywords

Snail; neuroendocrine differentiation; prostate cancer

Introduction

Prostate cancer, a second leading cause of death in men in the U.S. with metastasis primarily to bone (1–3). Epithelial-mesenchymal transition (EMT) is one mechanism by which tumor cells become more motile, invasive and metastatic (4–7). EMT is characterized by a switch from clustered cobblestone epithelial morphology, to loosely associated spindle-shaped mesenchymal cells. This is accompanied by an elevation of mesenchymal-associated genes, such as vimentin, N-cadherin and fibronectin, and a decrease in expression of epithelial-associated markers such as E-cadherin and cytokeratins (8–10).

Snail transcription factor, a member of the Snail superfamily, is a zinc finger protein that can inhibit E-cadherin by binding several E-boxes located in the promotor region (8). Snail transfection in Madine Darby canine kidney (MDCK) epithelial cells resulted in an EMT characterized by loss of E-cadherin expression and increased expression of vimentin (11), with concomitant increase in cell migration, invasion, and tumorigenesis. Snail can also repress tight junction proteins like Claudin, Occludin, and Zona Occludin-1 (12,13), and other epithelial genes such as Cytokeratin 18 and Mucin 1 (14). Snail has also been shown to confer survival properties through EMT-dependent or EMT-independent pathways (15–18). The subcellular localization and activity of Snail is controlled by its phosphorylation status (19–21). Glycogen synthase kinase-3 β (GSK-3 β) phosphorylates Snail and promotes its export from the nucleus and subsequent degradation by the proteasome in the cytosol, while p21-activated kinase (PAK1) can also phosphorylate Snail at a different residue from GSK-3 β to promote its nuclear localization and, thus, its activity as a transcription factor (20–22).

Neuroendocrine differentiation (NED) plays a role in both normal and pathological conditions of the prostate. The human prostate epithelial cells comprise of three cell types; secretory, basal and neuroendocrine (NE) cells (23). The NE cells release several secretory products that may act in an endocrine, paracrine, or autocrine manner in both normal and disease state (24). Two types of NE cell morphologies have been described; the “open” type resembling an open flask-shaped form with long slender extensions, and the “closed” type which lacks the extensions (25). However, the functions of the different cell types are unknown. The NE cell secretory products include serotonin, calcitonin, bombesin, neuron specific enolase (NSE), chromogranin A (CgA), and these regulatory products have been shown to induce tumor cell proliferation *in vitro* (26). NE cells appear to be quiescent, non-proliferative cells, that do not stain with the Ki-67 and MIB1 proliferation markers (27). Clinical studies have suggested that NED increases with tumor progression and the development of androgen refractoriness (28,29). Although more NE cells are observed in androgen-independent cells, it is unclear whether these cells actually induce androgen-refractoriness. NED has also been associated with tumors that are more aggressive and resistant to radiation, cytotoxic drugs and hormonal therapy (30–32). NE cancer cells are also more resistant to apoptosis (33). There fore, NE cells despite being quiescent can be implicated in tumor progression through secretion of mitogenic factors that maintain cell proliferation in adjacent tumor cells through a paracrine mechanism. Factors that can induce NED include androgen withdrawal, interleukin-6 (IL-6) cytokine, cyclic-AMP, and protocadherin-PC (27,34–38). Wu *et al.* showed that androgen withdrawal in LNCaP cells led to NED associated with activation of the phosphatidylinositol 3 kinase (PI3K)-AKT-mammalian target of rapamycin (mTOR) pathway, and extracellular signal-regulated kinase (ERK), although only the PI3K pathway was required for NED, as treatment with PI3K inhibitors prevented NED (39). Treatment of LNCaP cells with insulin growth factor-1 (IGF-1) also led to NED mediated by AKT activation (39). Wnt signaling was also activated in response to NED induction by protocadherin-PC (38). Signal transducers and activator of transcription (STAT3) has been shown to mediate IL-6-associated NED, while MAPK pathway was involved in the heparin binding-epidermal growth factor (HB-EGF)-mediated

NED (36,40). Androgen receptor (AR) has been shown to repress NED *in vitro* (41). Others have shown that NED is associated with decreased expression of AR and prostate specific antigen (PSA) *in vitro* (42,43). However, conditioned media from these NE cells stimulated proliferation and PSA secretion of parental LNCaP cells in androgen-deprived conditions by a paracrine mechanism (43). Additionally, *in vivo* experiments showed that LNCaP tumors from castrated mice bearing both LNCaP and NE-10 (a NE mouse prostate allograft), expressed increased levels of nuclear AR and PSA secretion as compared to LNCaP tumors grown alone (44). This suggested that NED by production of paracrine factors, was associated with hormone refractory prostate cancer.

We previously reported that Snail transcription factor overexpressed in androgen-dependent LNCaP cells resulted in an EMT characterized by relocalization of E-cadherin protein and increase in vimentin protein (45). In this communication, we show that Snail not only induces EMT but also induced NED in LNCaP cells, characterized by increase in NSE and CgA expression, and increased cell proliferation in a paracrine manner. There was also relocalization of AR into the cell nucleus accompanied by increased PSA expression. These studies reveal that Snail can induce both EMT and NED in androgen-dependent LNCaP cells. The dual actions of Snail make it a possible biomarker for cancer progression and a promising therapeutic target for human prostate cancer.

Materials and Methods

Reagents and antibodies

RPMI was obtained from VWR Int., West Chester PA, while penicillin-streptomycin was from BioWhittaker, Walkersville, MD. The protease inhibitor cocktail was from Roche Molecular Biochemicals, Indianapolis, IN. Mouse monoclonal anti-human E-cadherin antibody was from BD Transduction Laboratories, Lexington, KY. Mouse monoclonal anti-human vimentin, mouse monoclonal anti-AR, goat polyclonal anti-PSA (sc-7638, for western blot) and mouse monoclonal anti-PSA (sc-7316, for immunofluorescence) antibodies were from Santa Cruz Biotechnology, Santa Cruz, CA. Mouse monoclonal NSE and CgA were from DAKO, Inc., Carpinteria, CA. The rat monoclonal anti-Snail and HRP-conjugated goat anti-rat antibodies were from Cell Signaling Technology, Inc., Boston, MA. Fetal bovine serum (FBS), G418 and mouse monoclonal anti-human actin antibody were from Sigma-aldrich, Inc., St Louis, MO. Charcoal/dextran treated FBS (DCC-FBS) was from Hyclone, South Logan, UT. HRP-conjugated sheep anti-mouse antibody and the Enhanced chemiluminescence (ECL) detection reagent were purchased from Amersham Biosciences, Buckingham, England. HRP-conjugated rabbit anti-goat antibody was from Zymed Laboratories Inc., San Francisco, CA. The cell titer 96 aqueous solution was from Promega Corporation, Madison, WI. The control and Snail smartpool siRNA were obtained from Dharmacon Inc., Chicago, IL. The Snail cDNA construct was kindly provided by Dr Mien-Chie Hung, University of Texas, Houston, TX.

Cells and culture conditions

The human prostate cancer LNCaP and PC-3 cell lines were obtained from ATCC, Manassas, VA. LNCaP-C33 cell line, a stable NED cell model derived by prolonged androgen deprivation of LNCaP cells, was a kind gift from Dr Ming-Fong Lin, University of Nebraska, NE (43). LNCaP cells were previously stably transfected with empty vector (Neo) or constitutively active Snail cDNA (45). MCF7 Snail-transfected cells were a kind gift from Dr Mien-Chie Hung, University of Texas, Houston, TX. All the cells were grown in RPMI supplemented with 10% fetal bovine serum and 1× penicillin-streptomycin (plus 400 µg/ml G418 for LNCaP-Neo, LNCaP-Snail and MCF7-Snail cells), at 37°C with 5% CO₂ in a humidified incubator.

Western blot analysis

Confluent cells were lysed in a modified RIPA buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 0.02% Na₃N, 0.1% SDS, 1% NP-40, 0.5% sodium deoxycholate) containing 1.5× protease inhibitor cocktail, 1 mM phenylmethylsulfonyl fluoride, and 1 mM sodium orthovanadate. The cell lysates were centrifuged, and supernatants collected and quantified using a micro BCA assay. 30 µg of cell lysate was resolved on a 4–12% SDS PAGE, followed by transblotting onto nitrocellulose membrane (Schleicher & Schuell, Keene, NH). The membranes were blocked in TBS-TB (TBS with 0.05% Tween-20, 0.05% BSA) containing 5% milk, and subsequently incubated with appropriate dilutions of antibody in blocking buffer. After washing, the membranes were incubated in peroxidase-conjugated sheep anti-mouse or rabbit anti-goat IgG, washed, and visualized using ECL reagent. The membranes were stripped using stripping buffer (Pierce Biotechnology, Inc., Rockford, IL) prior to reprobing with a different antibody.

Immunofluorescent analysis

2×10⁴ cells were plated onto chamber slides in T-medium containing 10% FBS and 400 µg/ml G418. After overnight culture, the cells were fixed with ice-cold methanol/acetone (1:1) followed by PBS rinse. Cells were blocked for 10 min with protein serum block (DAKO) followed by incubation with primary antibody for 1 h. Following rinsing with TBST, cells were incubated with Alexa-red secondary antibody (DAKO) for 1 h. After rinsing with TBST, slides were coverslipped and images were acquired using a laser-scan confocal microscope 410 (Carl Zeiss, Minneapolis, MN).

RNA isolation and RT-PCR

Total RNA was isolated from cells using the Qiagen kit as per manufacturer's instructions, and 1 µg reverse transcribed with oligo-dT using MMLV-reverse transcriptase (Invitrogen), to generate cDNA. PCR analyses were subsequently performed with 2 µl of cDNA utilizing the primers and conditions as follows: E-cadherin primers were 5'-TCCATTTCTTGGTCTACGCC-3' and 5'-CACCTTCAGCCAACCTGTTT-3'. The PCR conditions for E-cadherin were 95°C, 5 min, 35 cycles of 95°C, 30 s; 60°C, 30 s; 72°C, 60 s, and 72°C, 8 min final extension. Snail primers were 5'-TCA GAA TTC ATG CCG CGC TCT TTC CTC GTC AGG AAG CC 3' and 5' ACT GGA TCC TCA GCG GGG ACA TCC TGA GCA GCC GGA C 3'. Vimentin primers were 5'-TGGCACGTCTTGACCTTGAA-3' and 5'-GGTCATCGTGATGCTGAGAA-3'. The PCR conditions for vimentin and Snail were 94°C, 2 min, 29 cycles of 94°C, 30 s; 55°C, 30 s; 72°C, 2 min, and 72°C, 7 min final extension.

MTS proliferation assay

3000 LNCaP-Neo or LNCaP-Snail cells were plated onto 96-well and 5 h later, cell titer 96 aqueous solution was added for 1 h as per manufacturer's instructions daily for up to 3 days. dishes overnight. The absorbance at 490 nm was then read. For proliferation using conditioned media, 3000 LNCaP or 2000 PC3 cells were plated onto 96-well dishes overnight. The following day, 25% of 48 h conditioned media derived from LNCaP-Neo1 or LNCaP-Snail1 were then incubated with the cells for 0–3 days, after which cell titer 96 aqueous solution was added for 1 h as per manufacturer's instructions. The absorbance at 490 nm was then read. For proliferation using conditioned media from LNCaP-Neo5 or LNCaP-Snail5 cells treated with NSE and/or CgA siRNA, 1000 LNCaP or 500 PC3 cells were plated onto 96-well dishes overnight. The following day, 25% conditioned media was then incubated with the cells for 0–4 days.

siRNA treatments

To induce NED in LNCaP cells, 2×10^4 cells were plated in 6-well dishes in T medium with 5% FBS, and after 24 h, the media was replaced with androgen-deprived medium (RPMI media containing 5% DCC-FBS) for 2–5 days. Subsequently, these cells were transfected with 200 nM control or Snail siRNA smartpool using Dharmafect I as per manufacturer's instructions (Dharmacon, Inc., Chicago, IL) for 3 days followed by total RNA or cell lysate preparation as described above. 2×10^4 LNCaP-C33 cells were plated in 6-well dishes overnight in RPMI with 10% FBS followed by transfection with 200 nM control or Snail siRNA smartpool for 7 days (on the third day, an additional 200 nm siRNA was added to the cells). Cell lysates were then prepared. 2×10^4 LNCaP-Snail cells were plated in 6-well dishes overnight in RPMI with 10% FBS followed by transfection with 200 nM control or NSE/CgA siRNA smartpool for 7 days (on the third day, an additional 200 nm siRNA was added to the cells). Cell lysates were then prepared.

Results

Snail Transcription Factor Induces EMT in LNCaP prostate cancer cells

LNCaP cancer cells were generated previously by transfection with lipofectamine 2000, which overexpressed Snail transcription factor (45). Individual clones selected by culturing in media containing G418 showed that LNCaP-Snail1 clone displayed a neuroendocrine morphology when compared to LNCaP-Neo1 clone (Fig. 1A). Additionally, LNCaP-Snail1 clone expressed detectable levels of Snail compared to the mixed clone (LNCaP-Snail mix) or empty vector control (LNCaP-Neo1), and also expressed more vimentin protein by western blot and immunofluorescent analyses (Fig. 1B, C). We also confirmed the previous report that E-cadherin protein levels did not change significantly between LNCaP-Neo1 and LNCaP-Snail1 at the western blot level, but the localization differed; whereas E-cadherin was at cell-cell junctions in LNCaP-Neo1 cells, it was predominantly in the cytosol in LNCaP-Snail1 cells (Fig. 1B, C). Retransfection of LNCaP cells and generation of more clones showed that Snail clones generally expressed more Snail than the Neo controls, with no significant changes in E-cadherin levels, by Western blot analysis (supplementary Fig. 1A). This was associated with increased cell migration and relocalization of E-cadherin and β -catenin from the cell membrane to the cytosol, as well as increased vimentin staining as shown by immunofluorescent analysis (supplementary Fig. 1B). This confirms that Snail transcription factor induces EMT in LNCaP cells.

Snail induces NED in LNCaP prostate cancer cells

The morphology observed in LNCaP cells overexpressing Snail did not resemble the spindle like morphology typical of EMT, but rather long neurite-like processes reminiscent of a neuroendocrine phenotype (Fig. 1A). We therefore investigated whether the cells expressing Snail have undergone NED by examining for the NED markers neuron specific enolase (NSE) and chromogranin A (CgA). LNCaP cells transfected with Snail displayed increased expression of NSE and CgA by RT-PCR, western blot and immunofluorescence analyses (Fig. 2A, B, supplementary Fig. 1C). In addition, androgen receptor (AR) translocation to the nucleus was poignant in the cells transfected with Snail (Fig. 2B), while prostate specific antigen (PSA) levels appeared to increase by RT-PCR and western blot analyses (Fig. 2A). Quantification of NSE, CgA, AR and PSA Western blot levels showed that these proteins were increased (Fig. 2A, lower figure). These results indicate that Snail can induce NED accompanied by nuclear translocation of AR and increased PSA.

Snail inhibits cell proliferation in LNCaP cells but increases paracrine cell proliferation

Previous reports have indicated that NED is usually associated with reduced cell proliferation, although the function of these quiescent cells is to secrete growth factors that will increase cell proliferation of neighboring cells (26,27,43,44). So we examined cell proliferation using the MTS assay. We found that Snail does inhibit cell proliferation in LNCaP cells significantly within 3 days (Fig. 3A). Functionally, although the LNCaP-Snail1 cells were growth-arrested, the conditioned media from these cells were able to induce more cell proliferation in parental androgen-dependent LNCaP and androgen-independent PC3 cells, than conditioned media from LNCaP-Neo1 cells when tested using the MTS assay (Fig. 3B). This suggests that factors produced by LNCaP-Snail1 cells can act in a paracrine manner to promote cell proliferation of cancer cells in the vicinity. Therefore, the Snail expressed in LNCaP cells is functional and can induce paracrine cell proliferation.

Snail inhibition can abrogate NED induced by androgen deprivation

Next, we examined whether Snail would regulate NED induced by a different mechanism. It is well documented that androgen deprivation will induce NED (43). We induced NED in LNCaP cells by growth in androgen-depleted media (5% DCC) for 7 days. This was then followed by treatment with either control siRNA or Snail siRNA for an additional 3 days. As compared to growth in 10% FBS, 5% DCC induced NED as evidenced by increased NSE at the level of RT-PCR and western blot analyses, although CgA was not induced by 5% DCC within this time point (Fig. 4A). However, treatment with Snail siRNA led to a reduction in NSE and CgA by both RT-PCR and western blot (Fig. 4A). These results were confirmed by quantifying the Western blot results relative to actin levels (Fig. 4B). We also utilized LNCaP-C33 cells, an established stable NED model that was previously derived from long-term passage in androgen-free media (43). We found that Snail expression is higher in LNCaP-C33 cells compared to LNCaP cells and confirmed that these cells do express higher levels of NSE and CgA by western blot analysis (Fig. 5A). Knockdown of Snail in LNCaP-C33 cells resulted in about 50% reduction in Snail protein after 7 days, as well as reduction in NSE and CgA protein levels as seen by Western blot analysis and quantification of these proteins relative to actin levels. (Fig. 5B). An MTS assay showed that conditioned media from LNCaP-C33 treated with Snail siRNA led to a significant decrease in cell proliferation in parental LNCaP (Fig. 5C) and PC3 (Fig. 5D) cells by day 3, as compared to conditioned media from LNCaP-C33 cells treated with control siRNA. Therefore, Snail does regulate NED induced by androgen deprivation in LNCaP cells, and knockdown of Snail can abrogate NED in these cells.

Antagonizing NSE and CgA in LNCaP-Snail abrogates paracrine cell proliferation induced by LNCaP-Snail cells

In order to examine whether NSE and CgA secreted by LNCaP-Snail cells contribute to paracrine cell proliferation, we treated LNCaP-Snail 5 cells with 200 nM control siRNA or 200 nM NSE and/or CgA siRNA for 7 days. Western blot analysis on the cell lysates prepared from these cells and quantification of the Western blot showed that NSE or NSE/CgA siRNA efficiently knocked down NSE protein levels by approximately 60% or 75%, respectively, while CgA siRNA or NSE/CgA siRNA led to knock down of CgA protein by about 40% or 60%, respectively (Fig. 6A,B). Interestingly, NSE and/or CgA siRNA also led to at least a 50% decrease in Snail protein levels (Fig. 6A, B). Conditioned media from these cells indicated that while LNCaP-Snail5 CM increased proliferation of parental LNCaP and PC3 cells as compared to LNCaP-Neo5 cells, this was abrogated by treatment with NSE and/or CgA siRNA as compared to control siRNA (Fig. 6C, D). This suggests that NSE and CgA contributes to paracrine cell proliferation induced by Snail in LNCaP cells.

Discussion

The epithelial-mesenchymal transition (EMT), initially described by developmental biologists as the morphological changes that epithelial cells undergo at specific sites during embryonic development, resulting in more migratory cells, has been found to occur in epithelial tumor cells (4–7). This involves loss of epithelial characteristics and gain of mesenchymal characteristics as the cells become more invasive, migratory, and metastatic (8–10). In prostatic carcinoma, neuroendocrine differentiation (NED) involves the transition of prostate cancer cells into neuroendocrine-like cells that express neuroendocrine markers like neuron specific enolase (NSE) and chromogranin A (CgA), and is associated with tumor progression to a hormone refractory stage, and poor prognosis. Factors secreted by these neuroendocrine cells are believed to have mitogenic effects on adjacent cells and contribute to androgen independent proliferation of prostate cancer cells (24,46,47). NED has also been observed in metastatic lesions and correlate significantly to prostate cancer mortality (48,49). Our present study shows for the first time that Snail transcription factor, that normally induces EMT, can also induce NED, thus providing a link between EMT and NED.

This study and a previous study has shown that overexpression of Snail in LNCaP prostate cancer cells led to an EMT characterized redistribution of E-cadherin protein from the cell surface to the cytosol, increase in vimentin protein, and increased cell migration (45). Additionally, Snail induced a neuroendocrine-like morphology in LNCaP cells. Increased expression of NSE and CgA in these cells confirmed that Snail did induce NED in LNCaP cells. The NED induced by Snail was functional leading to increased proliferation of parental LNCaP and PC3 prostate cancer cells by a paracrine mechanism. LNCaP cells that underwent NED due to androgen withdrawal for up to 7 days, or the LNCaP-C33 NED prostate cancer cell model generated previously by androgen withdrawal long term (43), also displayed increased Snail expression. NED was abrogated in LNCaP-C33 cells by Snail knockdown with siRNA. These experiments confirm that Snail is indeed able to regulate NED induced by either Snail overexpression or androgen withdrawal. We also found that Snail-induced NED and paracrine cell proliferation can be abrogated by knocking down NSE and/or CgA with siRNA, which suggests that these neuroendocrine proteins secreted in response to Snail, contribute to paracrine cell proliferation. It was interesting to note that silencing these neuroendocrine markers led to silencing of Snail protein, which suggests that NSE and CgA may also be able to regulate Snail protein. To our knowledge this is the first report showing that Snail can regulate NED.

Interestingly, we found that Snail induced translocation of AR to the nucleus and increased expression of PSA. A previous study has shown that neuroendocrine secretions can activate AR transcriptional activity via the NF κ B pathway in LNCaP cells (50). In our study Snail, may either be directly activating AR, or via neuropeptides secreted that may act in an autocrine manner. Further experiments are required to elucidate whether Snail can transactivate the AR promoter. Androgen ablation therapy for treatment of advanced prostate cancer usually progresses to a hormone refractory state (51). It has been noted that even in the hormone refractory cancer, the AR signaling axis is still intact, although paradoxically AR protein expression has been shown to prevent NED (52,53). Another study showed that short-term exposure of LNCaP cells to interleukin-6 (IL-6) led to NED, while long-term exposure to IL-6 led to AR activation (54). Future studies will look at whether Snail transcription factor may contribute to tumor progression from androgen-dependent to hormone refractoriness by inducing NED and AR activation.

Collectively, our results show for the first time that Snail transcription factor provides a link between EMT and NED in LNCaP prostate cancer cells. Our data underscores the potential

in future of utilizing Snail as a diagnostic marker for tumor aggressiveness and a future target for therapy against prostate cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Grant supported by: PC073405 and 2G12RR003062-22 to VOM.

Abbreviations

NED	neuroendocrine differentiation
EMT	epithelial mesenchymal transition
NSE	neuron specific enolase
CgA	chromogranin A

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Figure 1. Snail transcription factor induces EMT in LNCaP cells

(A) LNCaP prostate cancer cells stably transfected with Snail (Snail1 clone) displayed a neuroendocrine morphology when compared with LNCaP cells transfected with empty vector (LNCaP-Neo1). (B) LNCaP-Neo1 or -Snail1 were plated overnight onto 16-well chamber slide, fixed with methanol/acetone and examined by immunofluorescent analysis for E-cad, Vimentin or secondary antibody alone negative control. Mag. X200. (C) LNCaP prostate cancer cells stably transfected with either empty vector (LNCaP-Neo1) or Snail transcription factor (Snail mix or Snail1 clone) were examined for expression of Snail EMT markers, E-cadherin (E-cad) and vimentin by Western blot analysis. Actin was utilized as a loading control. Quantification of the Western blot results by densitometry with normalization to actin levels was done using the Quantity One quantification software (BioRad). All the experiments were repeated at least three times.

Figure 2. Induction of NED and AR nuclear translocation by Snail transcription factor
 (A) Total RNA or cell lysate was isolated from the LNCaP-Neo1 and LNCaP-Snail1 cells, and analyzed by RT-PCR or Western blot using primers or antibodies, respectively, for NED markers Neuron Specific Enolase (NSE), chromogranin A (CgA), as well as AR and PSA. 18SrRNA or actin was used as a loading control. Quantification of the Western blot results by densitometry with normalization to actin levels was done using the Quantity One quantification software (BioRad). (B) Immunofluorescence with specific antibodies revealed increased expression of NSE and CgA and AR translocation to the nucleus in LNCaP-Snail1 cells when compared to LNCaP-Neo1 cells. Mag. X100. All results shown are representative of three independent experiments.

Figure 3. Snail inhibits cell proliferation in transfected LNCaP cells, but conditioned media from Snail-transfected cells can increase cell proliferation of parental LNCaP and PC3 cells by a paracrine mechanism

(A) LNCaP-Neo1 and LNCaP-Snail1 or LNCaP-Neo5 and LNCaP-Snail5 clones were plated onto 96-well plates and MTS assay performed for 0–3 days. Cells transfected with Snail showed decreased cell proliferation. (B) Parental LNCaP or PC3 cells were plated overnight onto 96-well plates, followed by replacement of media with 25% conditioned media from either LNCaP-Neo1 or LNCaP-Snail1 cells. The MTS assay was then performed for 0–3 days. All experiment were done in triplicate and repeated twice independently. Bars, SD *, $P < 0.05$, Student's t test compared with LNCaP-Neo1 CM.

Figure 4. NED induced in LNCaP cells by androgen deprivation can be abrogated by Snail siRNA

(A) LNCaP cells were plated in 10% FBS overnight then cultured in either 10 % FBS or androgen-deprived media (5% DCC) for 7 days. The cells in DCC were then cultured for an additional 3 days in either 5% DCC plus 200 nM control siRNA or 5% DCC plus 200 nM Snail siRNA. Total RNA and cell lysates were prepared and analyzed by RT-PCR and western blot analyses for Snail, NSE and CgA. GAPDH or actin were utilized as loading controls for RT-PCR or western blot, respectively. (B) Quantification of the Western blot results by densitometry with normalization to actin levels was done using the Quantity One quantification software (BioRad).

Figure 5. NED in the LNCaP-c33 cell model is mediated by Snail transcription factor

(A) LNCaP-C33 cells that are NED cells derived by prolonged androgen deprivation, were analyzed for expression of Snail and the NED markers NSE and CgA utilizing western blot analysis. As compared to LNCaP cells, there was higher expression of Snail, NSE and CgA protein. (B) LNCaP-C33 cells were treated with control of Snail siRNA for 7 days, which resulted in approximately 50% knockdown of Snail, NSE and CgA protein as seen by Western blot analysis. Quantification of the Western blot results by densitometry with normalization to actin levels was done using the Quantity One quantification software (BioRad). The conditioned media from LNCaP-C33 cells treated for 7 days with either control or Snail siRNA was collected and added to (C) parental LNCaP, or (D) PC3 cells whose cell proliferation was monitored for up to 3 days. All experiments were done in triplicate and repeated twice independently. Bars, SD *, $P < 0.05$, Student's t test compared with control siRNA.

Figure 6. Paracrine cell proliferation induced in LNCaP and PC3 parental cells by LNCaP-Snail conditioned media can be abrogated by NSE and CgA siRNA

(A) LNCaP-Snail5 cells were plated in 10% FBS overnight then transfected with either 200 nM control siRNA or 200 nM NSE and/or CgA siRNA for 7 days. Untreated LNCaP-Neo5 and LNCaP-Snail5 were included as controls. Total cell lysates were prepared and analyzed by western blot analyses for Snail, NSE and CgA. Actin was utilized as a loading control for western blot. (B) Quantification of the Western blot results by densitometry with normalization to actin levels was done using the Quantity One quantification software (BioRad). The conditioned media from untreated LNCaP-Neo5, untreated LNCaP-Snail5 and LNCaP-Snail5 cells treated for 7 days with either control or NSE/CgA siRNA was collected and added to (C) parental LNCaP, or (D) PC3 cells whose cell proliferation was monitored for up to 4 days. All experiments were done in triplicate and repeated twice independently. Bars, SD, $P = 0.069$ for LNCaP treated with NSE+CgA siRNA CM for 4 days, $P = 0.058$ for PC3 treated with, NSE+CgACM for 4 days; Student's t test compared with control siRNA CM.