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Snail-mediated regulation of reactive oxygen species in ARCaP human prostate cancer cells

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ABSTRACT

Reactive oxygen species increases in various diseases including cancer and has been associated with induction of epithelial–mesenchymal transition (EMT), as evidenced by decrease in cell adhesion-associated molecules like E-cadherin, and increase in mesenchymal markers like vimentin. We investigated the molecular mechanisms by which Snail transcription factor, an inducer of EMT, promotes tumor aggressiveness utilizing ARCaP prostate cancer cell line. An EMT model created by Snail overexpression in ARCaP cells was associated with decreased E-cadherin and increased vimentin. Moreover, Snail-expressing cells displayed increased concentration of reactive oxygen species (ROS), specifically, superoxide and hydrogen peroxide, *in vitro* and *in vivo*. Real Time PCR profiling demonstrated increased expression of oxidative stress-responsive genes, such as aldehyde oxidase I, in response to Snail. The ROS scavenger, *N*-acetyl cysteine partially reversed Snail-mediated EMT after 7 days characterized by increased E-cadherin levels and decreased ERK activity, while treatment with the MEK inhibitor, UO126, resulted in a more marked effect by 3 days, characterized by cells returning back to the epithelial morphology and increased E-cadherin. In conclusion, this study shows for the first time that Snail transcription factor can regulate oxidative stress enzymes and increase ROS-mediated EMT regulated in part by ERK activation. Therefore, Snail may be an attractive molecule for therapeutic targeting to prevent tumor progression in human prostate cancer.

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1. Introduction

Epithelial–mesenchymal transition (EMT) is one mechanism by which tumor cells become more motile, invasive and metastatic [1–4]. EMT is characterized by an upregulation 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 [5–7]. Snail transcription factor, a member of the Snail superfamily, is a zinc finger protein that can induce EMT characterized by loss of E-cadherin expression and increased expression of vimentin, with concomitant increase in cell migration, invasion, and tumorigenesis [8].

It is well recognized that human cancer development is associated with chronic inflammation, and ROS released by inflammatory cells may result in DNA damage [9,10]. It has also been reported that spontaneous generation of ROS in tumor tissue was positively correlated with clinical stage in small cell lung cancer and squamous cell carcinoma patients [11]. ROS has also been associated with EMT; TGF-β was shown to induce EMT *via* up-regulation of hydrogen peroxide and MAPK ERK signaling in proximal tubular epithelial cells [12], while MMP-3 mediated EMT in mammary epithelial cells involved increase in ROS and Snail [13].

We have previously established an ARCaP human prostate cancer EMT cell model by overexpression of Snail transcription factor [14,15]. Utilizing this model, we have found that Snail-mediated EMT is partly regulated by ROS and ERK signaling in prostate cancer cells. Additionally, the hydrogen peroxide scavenger NAC, and MAPK inhibitor, UO126, could partially revert EMT.

2. Materials and methods

2.1. Reagents and antibodies

RPMI 1640 medium (1× with L-glutamine and without L-glutamine and phenol red medium) and penicillin–streptomycin were

Abbreviations: EMT, epithelial mesenchymal transition; ROS, reactive oxygen species.

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from Mediatech (Manassas, VA). 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 and ERK1 antibodies were from Santa Cruz Biotechnology, Santa Cruz, CA. MEK inhibitor UO126, *N*-acetyl cysteine (NAC), and mouse monoclonal anti-human actin antibody were from Sigma–Aldrich, Inc., St. Louis, MO. G418 was from EMD Corp BioScience (Brookfield, WI). Rat monoclonal anti-human Snail antibody, rabbit polyclonal anti-phospho-ERK antibody and HRP-conjugated goat anti-rat antibody were from Cell Signaling Technology, Inc., Danvers, MA. HRP-conjugated sheep anti-mouse, sheep anti-rabbit and the enhanced chemiluminescence (ECL) detection reagent were purchased from Amersham Biosciences, Buckingham, England. Fetal bovine serum (FBS) and charcoal/dextran treated FBS (DCC-FBS) were from Hyclone, South Logan, UT. Dihydroethidium bromide (DHE) and dichlorofluorescein (CM-DCFDA) were obtained from Invitrogen, Carlsbad, CA.

2.2. Cell Lines and Culture

ARCaP cells stably transfected with constitutively active Snail cDNA has been described previously for ARCaP-Neo6,8 and ARCaP-Snail11, 12, 13, 14 [14]. Cells were grown in RPMI supplemented with 10% fetal bovine serum and 1× penicillin–streptomycin, at 37 °C with 5% CO_2 in a humidified incubator.

2.3. Western blot analysis

Western blot was performed as described previously [14]. The membranes were stripped using stripping buffer (Pierce Biotechnology, Inc., Rockford, IL) prior to re-probing with a different antibody. For treatments, 70% confluent cells were serum-starved in phenol red-free serum-free RPMI containing penicillin/streptomycin for 24 h prior to treatment with NAC or UO126, in phenol-free serum-free RPMI containing 5% FBS DCC-FBS for 3–7 days.

2.4. Animal experiments

All of the animal procedures were approved and performed in accordance with Emory University Institutional IACUC guidelines. Four-week-old male athymic *nu/nu* mice (National Cancer Institute) were injected subcutaneously with 2×10^6 cells per mouse of Neo or Snail-overexpressing ARCaP cells mixed 1:1 volume with matrigel (BD Biosciences). The mice were sacrificed after 5–10 weeks, the tumors excised and tumor volume measured with a caliper (tumor volume was calculated as 3.14/6 × largest diameter × smallest diameter squared). Half the tumor was used for histology studies while the other half was used for *in vivo* ROS studies as outlined below.

2.5. In vitro and in vivo measurement of ROS with DHE or DCF

For *in vitro* experiments, 70% confluent cells were washed with PBS followed by trypsin digestion. Cells were pelleted at 300 g for 2 min, the supernatant removed and the cells resuspended in 500 μL of HANKS with 5% FBS. Cells were split into two aliquots of 250 μL each, and either 2 μM CM-DCFDA (to detect hydrogen peroxide) or 10 μM DHE (to detect superoxide) was added to cells, followed by incubation for 30 min while gently rocking in the dark. Cells (20,000) were gated and analyzed by Fluorescence Activated Cell Sorting (FACS). For *in vivo* experiments, freshly harvested tissue was place in OCT and frozen on dry ice. Tissues were immediately sliced to a thickness of 10 μm, placed on glass slides, and kept frozen on dry ice for an additional 2 h. After rapid thaw, tissues were treated with HANKS alone, 10 μM ebselen (Sigma) in HANKS (final 0.02% ethanol) or 1000 units SOD-PEG (Sigma) in HANKS for 20 min at 37 °C. Immediately, cells were incubated in the dark with either 2 μM CM-DCFDA in HANKS (0.25% DMSO) with TOPRO III (Invitrogen), or 10 μM DHE in HANKS (0.25% DMSO) at 37 °C. Slides were washed in 1× PBS for 5 min followed by mounting with Vectashield (Hardset) (Vector Labs). Fluorescence was visualized immediately by confocal microscopy (Ziess LMS 510 Meta).

2.6. PC arrays

Total cellular RNA was isolated from ARCaP-Neo or ARCaP-Snail cells that were about 80% confluent, using Rneasy Mini Kit (Quiagen). Integrity of isolated total cellular RNA was verified by Agilent Bioanalyser 2100 using RNA 6000 Pico Chip (18S and 28S ribosomal RNAs displayed sharp peaks without shoulders in RNA samples from both cell types and the ratio of 28S:18S rRNAs were 1.73 and 2.2 for ARCaP-Snail and ARCaP-Neo cells, respectively). RNA (1 μg) (determined by Nanodrop) was reverse transcribed using RT^2 First Strand Kit (SABiosciences, Cat. No. C-03) following manufacturer's protocol (20 μL reaction) and diluted with 91 μL water (Molecular Biology grade). Diluted cDNA was used to perform RT^2 Profiler PCR Array (Human Oxidative Stress and Antioxidant Defense, SABiosciences, Cat. No. PAHS-065C-2) in ABI StepOnePlus Real Time PCR System (SABiosciences) following manufacturer's protocol. This PCR Array profiles expression of 84 genes related to oxidative stress. Raw Ct values for individual genes were converted to fold change results (ARCaP-Snail:ARCaP-Neo) using RT^2 PCR Array Data Analysis portal (http://www.SABiosciences.com/pcrarraydataanalysis.php). GAPDH was chosen as an appropriate endogenous control gene for expression data normalization based on prior validation experiments (data not shown). Genes with up- or down-regulation $\geqslant 2.5$ or $\leqslant -2.5$ were considered as significantly differentially expressed between ARCaP-Snail and ARCaP-Neo cells.

3. Results

3.1. ROS is elevated in vitro and in vivo in ARCaP cells transfected with snail

We utilized ARCaPE cells overexpressing Snail described previously [14] that displayed increased Snail and vimentin expression, and decreased E-cadherin, as compared to Neo control cells (Fig. 1A) Because ROS-Snail signaling has been implicated with breast cancer [13], we examined *in vitro* ROS levels in the ARCaP prostate cancer cell lines transfected with Snail. Using dihydroethidium (DHE) staining for superoxide and dichlorofluorescein (DCF) staining for hydrogen peroxide, we found elevated levels of superoxide and hydrogen peroxide in ARCaPE cells transfected with Snail (ARCaP-Snail12) *in vitro* as compared to the control (ARCaP-Neo8) (Fig. 1B). As elevated levels of ROS were observed in prostate cancer cell lines expressing Snail *in vitro*, we next investigated whether Snail overexpression can increase levels of ROS *in vivo*. ARCaP-Neo8 and -Snail12 transfected cells were grown subcutaneously in nude mice and sacrificed after 10 weeks. Mice bearing ARCaP-Snail12 cells bore significantly larger tumor volumes as compared to ARCaP-Neo8 mice (Fig. 1C), and the presence of tumor cells was confirmed by H&E staining (Fig. 1D). Excised tumors were analyzed for ROS *in vivo* utilizing DHE stain for superoxide and DCF-DA stain for hydrogen peroxide, respectively. This revealed increased staining for both superoxide and hydrogen peroxide in ARCaP-Snail bearing tumors compared to ARCaP-Neo (Fig. 1E). This staining could be partly blocked by treatment with the superoxide scavenger, SOD-PEG, or the hydrogen peroxide scavenger, ebselen, as compared to control treatment with Hanks medium (Fig. 1E).

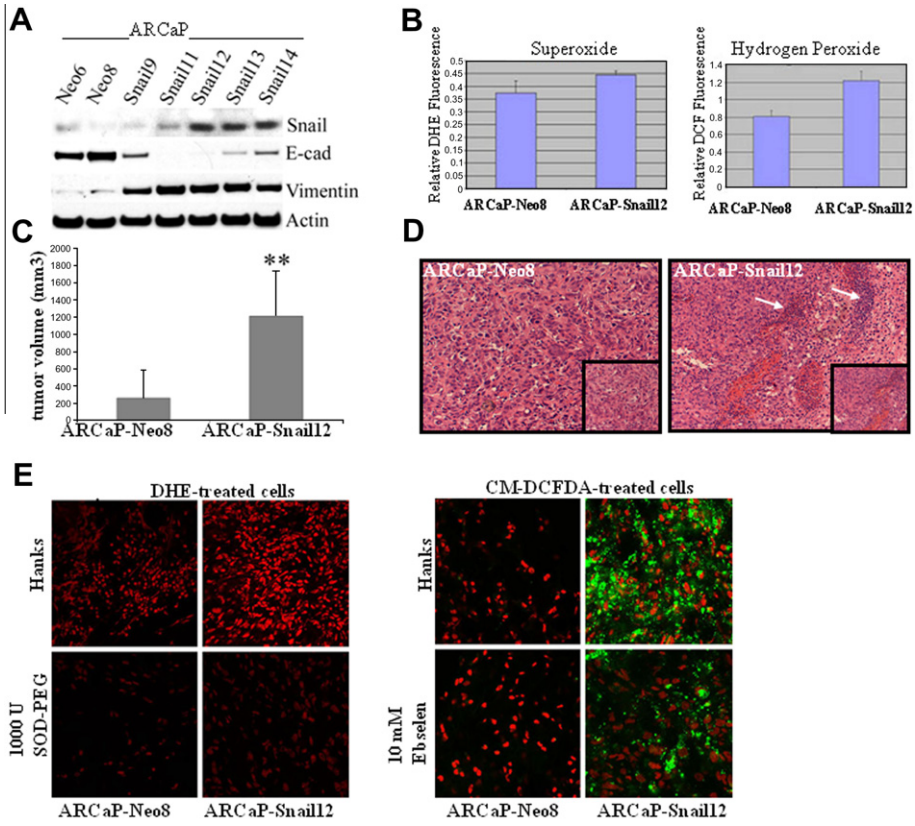

Fig. 1. ROS is increased *in vitro* and *in vivo* in ARCaP prostate cancer cells overexpressing Snail. (A) ARCaP clones transfected with Snail cDNA was confirmed to undergo EMT as shown by increased Snail and vimentin, and decreased E-cadherin by Western blot analysis, as compared to ARCaP cells transfected with empty vector (Neo6, Neo8). (B) Representative ARCaP-Neo8 and ARCaP-Snail12 were tested for *in vitro* ROS using DHE stain for superoxide and DCF stain for hydrogen peroxide. (C) 2×10^6 ARCaP cells transfected with Snail (ARCaP-Snail12) or empty vector (ARCaP-Neo8) were injected subcutaneously into eight nude mice and sacrificed after 10 weeks. Tumor volumes measured indicated that tumors from ARCaP-Snail12 were significantly larger than ARCaP-Neo8. (D) Tumors were excised, fixed in ethanol and stained with hematoxylin and eosin to visualize tumor cells. (E) Excised tumors were flash frozen and stained with 10 μM DHE to measure superoxide levels *in vivo* and specificity of stain measured by co-treatment with control Hanks solution or 1000U SOD-PEG to scavenge superoxide. Increased superoxide was observed in the Snail-expressing tumors (ARCaP-Snail12) as compared to ARCaP-Neo8. Flash frozen tumors were also stained with 2 μM CM-DCFDA to measure hydrogen peroxide levels *in vivo*. Increased levels of hydrogen peroxide were observed in ARCaP-Snail12 compared to ARCaP-Neo8 tumors, which could be inhibited by treatment with the hydrogen peroxide scavenger, ebselen (10 μM). Results are representative of three independent animal experiments. Magnification ×100; inset ×200. Data represent mean ± SD ($^{**}p < 0.005$).

These results show that Snail overexpression leads to increased hydrogen peroxide and superoxide, *in vitro* and *in vivo* in human xenografted ARCaP prostate cancer cells.

3.2. Snail overexpression in ARCaP cells regulates oxidative stress-responsive genes

In order to determine the mechanism by which Snail could increase ROS, we examined expression of oxidative stress genes by using Real Time PCR Array. Genes identified as significantly differentially expressed between ARCaP-Snail and ARCaP-Neo cells are listed in Table 1. The results for all genes present in the Human Oxidative Stress and Antioxidant Defense PCR Array are provided in Supplementary Fig. 1. Snail led to up-regulation of several oxidative stress enzymes, including aldehyde oxidase 1 (AOX1) which was increased 14.56-fold, and may be the most likely source of ROS as this molybdenum-containing enzyme oxidizes various aldehydes, including acetaldehyde and retinal, and produces ROS that promotes cell damage and fibrogenesis [16].

3.3. Antagonizing hydrogen peroxide with NAC or MAPK inhibitor leads to partial inhibition of EMT in ARCaP cells transfected with snail

We investigated whether treatment with the ROS scavenger, NAC would inhibit EMT. Since ERK is known to be downstream of ROS we also examined ERK activity and the effect of inhibiting ERK. Treatment with 5 or 10 mM NAC led to an increase in E-cadherin mRNA and protein level by 3 days and more prominently by 7 days (Fig. 2A and B). NAC also appeared to increase vimentin protein levels transiently at 3 days, although by 7 days the levels had decreased to levels comparable to untreated ARCaP-Snail cells (Fig. 2B and C). NAC did not affect Snail expression (data not shown). We also observed increased ERK activity due to Snail transfection, which was inhibited transiently by NAC at 3 days with return of full activity by 7 days (Fig. 2B). ERK activity was completely abrogated by treatment with the MEK inhibitor, UO126, concomitant with re-expression of E-cadherin to levels comparable to ARCaP-Neo8 cells within 7 days, although vimentin levels were not significantly changed (Fig. 2C). This suggests that antagonizing ROS or ERK activity can revert EMT partially by increasing E-cadherin protein levels that had been lost by Snail transfection.

4. Discussion

This study examined the molecular mechanism(s) by which Snail transcription factor may contribute to prostate cancer progression through regulation of ROS. There has been a report on increased hydrogen peroxide levels in human prostate tumors [17]. Since ROS has been shown to induce EMT and Snail [12,13],

Table 1
Snail can regulate oxidative stress-responsive genes. Total cellular RNA isolated from ARCaP-Neo or ARCaP-Snail cells was reverse-transcribed and Real Time PCR performed utilizing PCR Array profiles expressing 84 genes related to oxidative stress. Raw Ct values for individual genes were converted to fold change results (ARCaP-Snail:ARCaP-Neo) using RT^2 PCR Array Data Analysis portal (http://www.SABiosciences.com/pcrarraydataanalysis.php) and GAPDH as the endogenous control gene.

Gene symbol	Gene name	Fold up- or down-regulation	Pathway/gene function
PXDN	Peroxidasin	353.29	Catalyzes hydrogen peroxide-driven radioiodination, oxidations, and the formation of dityrosine, extracellular matrix formation (Nelson et al. [31])
AOX1	Aldehyde oxidase 1	14.56	Produces ROS (Neumeier et al. [16])
SEPP1	Selenoprotein P, plasma, 1	8.54	Oxidative stress responsive gene (Burk and Hill [27])
PDLIM1	PDZ and LIM domain 1	6.08	Actin stress fiber formation (Tamura et al. [26])
CYGB	Cytoglobin	3.27	Scavenge nitric oxide or other reactive oxygen species, or serve a protective function during oxidative stress (Halligan et al. [29])
PNKP	Polynucleotide kinase-3′-phosphatase	2.84	Oxidative stress responsive gene (Jilani et al. [30])
SRXN1	Sulfiredoxin 1 homolog	2.7	Oxidative stress responsive gene (Rhee et al. [28])
PTGS2	Prostaglandin-endoperoxide synthase 2	−5.24	Key enzyme in prostaglandin biosynthesis, and acts both as a dioxygenase as a peroxidase (Otto and Smith [35])
NOS2	Nitric oxide synthase 2, inducible	−10.77	Induces nitric oxide reactive free radical which acts as a biologic mediator in several processes (Hevel et al. [34])

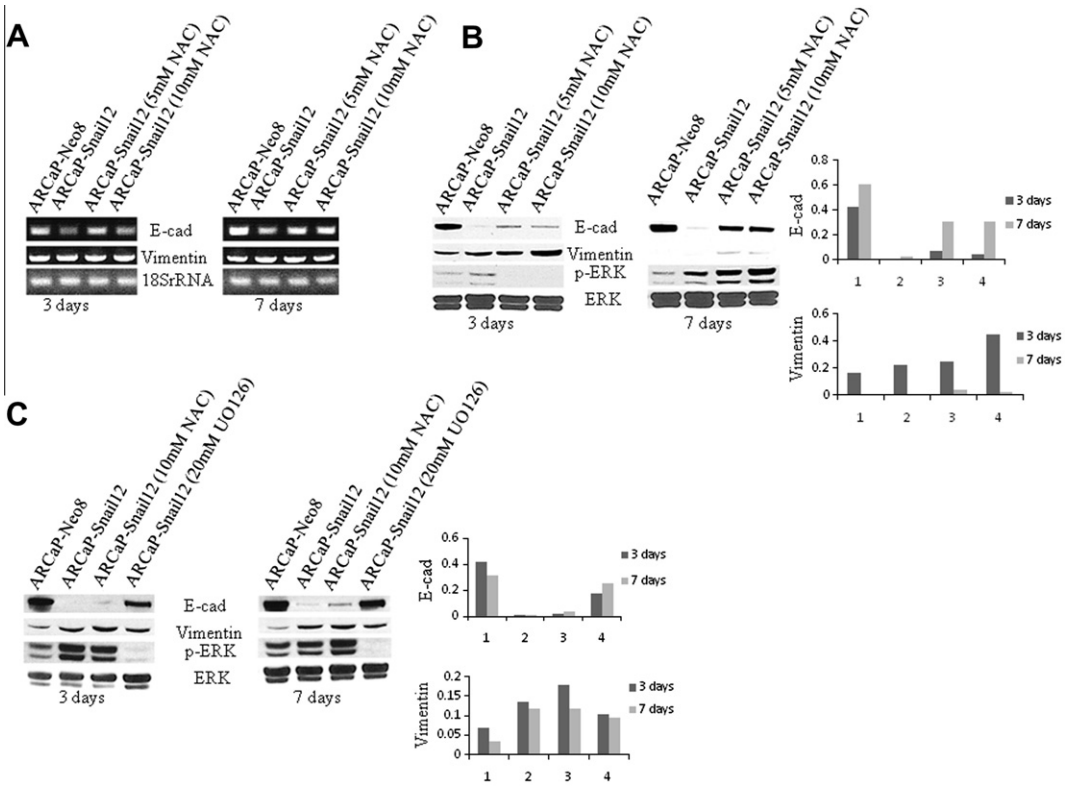

Fig. 2. *N*-Acetyl cysteine (NAC) or MAPK inhibitor (UO126) partially reverts EMT in ARCaP cells stably transfected with Snail. ARCaP-Snail12 cells were treated with 5 or 10 mM NAC for 3 or 7 days and E-cadherin (E-cad) and vimentin EMT markers analyzed by (A) RT-PCR and (B) Western blot analyses with quantification of protein performed for (1) ARCaP-Neo8, (2) ARCaP-Snail12, (3) ARCaP-Snail12 plus 5 mM NAC, and (4) ARCaP-Snail12 plus 10 mM NAC, in comparison to total ERK levels. In addition, ERK activity was examined utilizing polyclonal anti-phospho ERK antibody and compared to total ERK using monoclonal anti-ERK antibody. (C) Treatments of ARCaP-Snail12 with 10 mM NAC or 20 μM UO126 (MEK inhibitor) for 3–7 days followed by Western blot analysis revealed decreased ERK activity and increased E-cad expression, with the MEK inhibitor showing a more profound effect than NAC. Protein quantification is shown as compared to total ERK levels for (1) ARCaP-Neo8, (2) ARCaP-Snail12, (3) ARCaP-Snail12 plus 10 mM NAC, and (4) ARCaP-Snail12 plus 20 μM UO126.

we studied the possibility that the reverse is true; Snail can regulate ROS. Previously, hydrogen peroxide has been shown to increase progressively in the LNCaP sublines C4, C4-2, C4-2B that display increased tumorigenic and metastatic potential [17]. Increased hydrogen peroxide has also been observed in the prostate cancer cell lines LNCaP, DU145, PC3 and CL1 as compared to PNT1A normal prostate epithelial cell line [18]. One study showed that transfection of MMP-3 gene into breast cancer cells could induce both hydrogen peroxide and Snail [13].

In our study, ARCaPE cells transfected with Snail underwent EMT and the levels of hydrogen peroxide and superoxide increased in response to Snail *in vitro*. We further showed that subcutaneous injection of Snail transfectants led to increased tumorigenicity in the ARCaPE which is in agreement with a previous study by Peinado et al., which showed that Snail transfection into epithelial MDCK cell line led to an EMT characterized by increased tumorigenicity in nude mice [19]. The *ex-vivo* model further showed that excised tumors from Snail-transfected ARCaP cells displayed increased hydrogen peroxide and superoxide. Therefore, both *in vitro* and *in vivo* data confirmed the novel finding that Snail was able to increase hydrogen peroxide and superoxide levels in ARCaP cells.

We also attempted to identify the possible source(s) of increased ROS concentration in ARCaP-Snail cells by Real Time PCR Array, and our data suggested aldehyde oxidase 1 (AOX1) that was upregulated 14.56-fold, as a likely source. This molybdenum-containing enzyme oxidizes various aldehydes, including acetaldehyde and retinal, which produces superoxide that promotes cell damage and fibrogenesis [16].

Overexpression of CCL5 (RANTES) gene by 2.48-fold in ARCaP-Snail cells (see Supplementary Fig. 1) is consistent with their migratory/invasive and metastatic phenotype. This inflammatory chemokine plays a crucial role in migration, invasion and metastasis of lung cancer [20], breast cancer [21,22], prostate cancer [23], chondrosarcomas [24] and other cancers by modulation of expression of αvβ3 integrin, and MMPs. In addition, the expression of CCL5 induced by TNF-alpha in murine mesangial cells was found to be contingent upon generation of ROS [25], which supports ROS-mediated expression of this gene. PDZ and LIM domain 1 (PDLIM1) gene that was upregulated 6.08-fold in ARCaP-Snail cells has been shown to promote stress fiber formation and focal adhesions in BeWo choriocarcinoma cells [26]. SRXN1 (2.7-fold upregulation), CYGB (3.27-fold upregulation) and SEPP1 (8.54-fold upregulation) function as antioxidants in intracellular or extracellular space and their overexpression in ARCaP-Snail cells is possibly associated with protective mechanisms of these cells against oxidative stress [27–29]. Similarly, upregulation of polynucleotide kinase 3′-phosphatase (PNKP), one of the most important enzymes responsible for the repair of oxidatively-induced DNA lesions [30], is likely associated with protection against ROS-mediated damage in ARCaP-Snail cells and is consistent with overproduction of ROS displayed by this cell line.

Interestingly, mammalian homolog of Drosophila peroxidasin (PXDN), a gene highly up-regulated in ARCaP-Snail cells, is a peroxidase that has been implicated in hydrogen peroxide-driven oxidations and extracellular matrix remodeling during TGF-β1-induced myofibroblast differentiation [31,32]. Importantly, myofibroblasts can also originate from renal tubular and endothelial cells that undergo epithelial to mesenchymal transition (EMT), and aberrant activation of Snail1 results in renal fibrosis in transgenic mice [33]. Therefore, it appears that molecular mechanisms responsible for disruption of epithelial homeostasis by ARCaP-Snail cells in cancer invasion and by myofibroblasts in fibrosis are very similar; associated with oxidative stress and Snail activity, and possibly also with secretion of PXDN and its incorporation into extracellular matrix. Thus, Snail-mediated EMT may represent a link between fibrosis and tumor progression.

NOS2 gene coding for nitric oxide (NO) – generating enzyme is associated with induction of nitric oxide [34], while PTGS2 acts as a dioxygenase and a peroxidase in prostaglandin biosynthesis [35], and down-regulation of NOS2 and PTGS2 may be part of protective mechanisms against oxidative stress.

We observed increased ERK activity in the ARCaP Snail transfectants. Indeed, ROS has been shown to induce ERK activity [12]. We found that treatment of the ARCaP Snail transfectants with the hydrogen peroxide scavenger, NAC, partially blocked EMT by inducing reexpression of E-cadherin and decreasing ERK activity, without affecting vimentin expression. This suggests that Snail may signal through ROS to increase ERK activity and EMT. In addition we found that inhibition of ERK with the MEK inhibitor, U0126, led to an even greater reexpression of E-cadherin protein to the level of the Neo control. We are investigating the possible mechanisms of how ERK may regulate E-cadherin. It is possible that inhibition of ERK may inhibit Snail transcription or Snail binding to the E-cadherin promoter. The former is more likely as Barbera et al. showed that inhibition of ERK activity in human mesenchymal cell lines, SW620 and MiaPaca, led to decreased Snail promoter activity and transcript levels, while constitutive activation of ERK resulted in greater Snail promoter activity [36]. This indicates that the ERK activity induced by Snail in our ARCaP cell model may result in a feedback loop to increase Snail transcription.

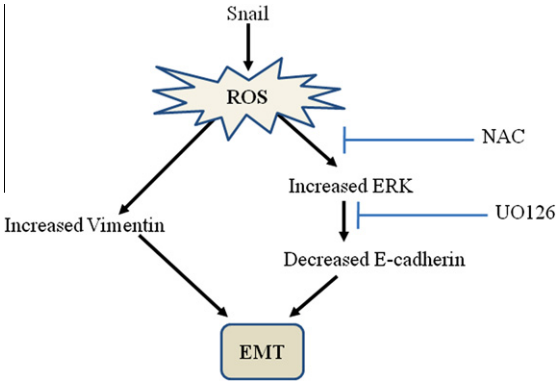

Fig. 3. Overall proposed mechanism of Snail regulation of EMT through ROS. We propose that Snail can turn on ROS that can induce EMT characterized by increased vimentin and decreased E-cadherin. NAC, a hydrogen peroxide scavenger, and UO126, MAPK inhibitor, can partially revert EMT by re-inducing E-cadherin without affecting vimentin.

Collectively, our results indicate that Snail can induce EMT through ROS signaling in ARCaP human prostate cancer cells, and may involve ERK signaling (Fig. 3). Snail may induce expression of antioxidant enzymes such as aldehyde oxidase I that may lead to increased levels of ROS. These studies show that a transcription factor, Snail, may mediate tumor progression through ROS signaling and underscores the importance of targeting these pathways with various inhibitors and antioxidants.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.11.044.

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