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Snail and Slug Mediate Radioresistance and Chemoresistance by Antagonizing p53-Mediated Apoptosis and Acquiring a Stem-Like Phenotype in Ovarian Cancer Cells

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ABSTRACT

The transcriptional repressors Snail and Slug contribute to cancer progression by mediating epithelial-mesenchymal transition (EMT), which results in tumor cell invasion and metastases. We extend this current understanding to demonstrate their involvement in the development of resistance to radiation and paclitaxel. The process is orchestrated through the acquisition of a novel subset of gene targets that is repressed under conditions of stress, effectively inactivating p53-mediated apoptosis, while another subset of targets continues to mediate EMT. Repressive activities are complemented by a concurrent derepression of specific genes resulting in the acquisition of stem cell-like characteristics. Such cells are bestowed with three critical capabilities, namely EMT, resistance to p53-mediated apoptosis, and a self-renewal program, that together define the functionality and survival of metastatic cancer stem cells. EMT provides a mechanism of escape to a new, less adverse niche; resistance to apoptosis ensures cell survival in conditions of stress in the primary tumor; whereas acquisition of "stemness" ensures generation of the critical tumor mass required for progression of micrometastases to macrometastases. Our findings, besides achieving considerable expansion of the inventory of direct genes targets, more importantly demonstrate that such elegant cooperative modulation of gene regulation mediated by Snail and Slug is critical for a cancer cell to acquire stem cell characteristics toward resisting radiotherapy- or chemotherapy-mediated cellular stress, and this may be a determinative aspect of aggressive cancer metastases. STEM CELLS *2009;27:2059–2068*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

The classic hallmarks of cancer include growth factor independence, insensitivity to antigrowth signals, evasion of programmed cell death, limitless replicative potential, sustained angiogenesis, and tissue invasion for metastasis [1]. Aggressive tumors selectively enhance these characteristics in progressing to a therapy-refractile state. Over the last decade, failure of cancer therapy has also been attributed to the persistence of quiescent cells called cancer stem cells (CSCs), that inherently express some of these characteristics, but are better recognized through their potential to efficiently regenerate tumor tissue even after therapeutic intervention [2]. The origin of these stem cells in tumors and their resistance to stress are not well delineated; however, they are proposed to arise either from transformation of stem and progenitor cells, or through dedifferentiation of cancer cells [3].

It is increasingly being realized that changes in cellular architecture and polarity affect cell fate of stem cells during development, organogenesis, and cancer. The generation of CSCs from immortalized human mammary epithelia has recently been shown to be triggered by one such process that mediates a change in cell morphology, namely epithelial mesenchymal transition (EMT) [4–6], that involves the loss of cell adhesion and acquisition of migratory and invasive properties by cells [7, 8]. However, the mechanistic understanding of the generation of CSCs through EMT has not yet been delineated, although an association is established.

The Snail family members Snail (*Snai1*) and Slug (*Snai2*) are closely related transcriptional repressors implicated in embryonic development, wherein they have been shown to be vital for the formation of mesoderm and neural crest through their recognized functionality of EMT [9]. In normal tissues of adult organisms, these transcription factors (TFs) are more commonly described in the context of wound healing, or protecting stem/progenitor cells from DNA damage [10]. Slug mediates radioprotective effects and enhanced survival of progenitor cells through activation of the SCF/cKit pathway [11, 12]. In the context of cancer, the current understanding of the

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role of these TFs focuses on their capability to mediate EMT. The acquisition of invasive properties by metastasizing cancer cells and disease progression to a stage associated with adverse prognosis for patients have been frequently attributed to the expression of *Snail* and *Slug* [13–15]. The well-established roles of Snail and Slug in EMT during both embryogenesis and tumor progression reflect their possible participation in migration of cancer stem cells [16]. EMT associated with recurrent wound healing has also been hypothesized as a risk factor in several physiological cancers, for example, in normal epithelial ovarian surface cell transformation [17], intestinal cell transformation [18], and others.

At the molecular level, Snail and Slug mediate gene regulation through recognition and specific binding to consensus sites in the promoter regions of their target genes that contain the core sequence -CANNTG- [19, 20]. This binding is followed by recruitment of factors involved in transcriptional repression including HDAC1, SIN3A, and so on [21]. Despite a strong implication of diverse roles of Snail and Slug in carcinogenesis, the currently known repertoire of direct targets of these TFs is, for the most part, restricted to genes encoding cell junction components. Moreover, since a majority of targets are reportedly repressed by both TFs, it is not known whether the latter play distinct or redundant roles during tumorigenesis; although circumstances suggest that Snail and Slug may modulate some of these targets differentially [12]. We reasoned that elucidation of the diverse functionality of these TFs may be addressed through identification of their complete repertoire of direct targets that could reflect the various processes possibly being regulated. Toward this aim, we applied several major genome-wide analyses: (a) in silico identification of the putative target genes of Snail and Slug in the human genome based on knowledge of the consensus sequence recognized by Snail and Slug, (b) introduction of TF specificity into the analyses by performing chromatin immunoprecipitation and promoter array analysis (ChIP-on-chip) using human genome-wide promoter arrays, and (c) screening of existing gene expression databases and published literature to identify possible additional targets. We thus derived a small subset of target genes that were further validated in a biological consequence, namely radioresistance and chemoresistance in ovarian cancer.

Our approach, besides achieving considerable expansion of the inventory of direct genes targets, and thereby, roles of these molecules in cancer, also suggests a definitive target gene modulation defining the molecular mechanisms mediated by Snail and Slug to overcome radiotherapy- or chemotherapy-mediated cellular stress. In a larger perspective, our findings reveal that these transcriptional repressors are critical for regulation of apoptosis and acquisition of stem-like characteristics by cancer cells.

Materials and Methods

In Silico Whole-Genome Analysis of Snail and Slug Binding Sites

The Human genome (build 36.1 reference assembly: ftp://ftp.ncbi.nih.gov/genomes/H_sapiens/ARCHIVE/BUILD.36.1) was used as the source for extracting the gene table information in conjunction with the gene sequences from FASTA formatted files; this constituted the primary database. We proceeded to extract a secondary database (consisting of 2-kb upstream to 1-kb downstream sequences from the transcription start site of all genes), and a tertiary database specific for the Snail and Slug binding site sequences, namely CACCTG and CAGGTG. A final list composed of 14,532 genes and corresponding number of E-boxes in their promoter regions was thus generated (supporting information Table 1, Fig. 1).

ChIP-on-chip and ChIP polymerase chain reactions

ChIP, combined with microarray analysis, was performed as described earlier [22]. The corresponding genes for the probes bound by Snail and/or Slug as identified through this effort are listed in supporting information Table 2. The calculation of percentage of target genes is based on the number genes with E-boxes in their promoter regions (5,677) of the total of 14,633 annotated genes on the arrays. Functional classification of these genes was carried out using gene expression analysis tool of PANTHER (Protein ANalysis THrough Evolutionary Relationships: http://www.pantherdb.org/tools/compareToRefListForm.jsp), which classifies genes by their functions; pathways indicated to be significant ($p < .005$) were identified and are represented in supporting information Figure 1. Promoter regions were amplified in immunoprecipitated chromatin by polymerase chain reaction (PCR) amplification using primers designed to specifically amplify the E-boxes within promoter regions of these genes; sequences are available on request. Amplified products were run on 1.8% agarose gel and band intensities measured by densitometric analysis using Genetool3.6 (Syngene, Cambridge, England, http://www.syngene.com).

Cell Culture and Establishment of Cell Systems

All cultures were maintained in minimum essential medium (E) plus 5% fetal calf serum and 1% nonessential amino acid solution at 37°C in 5% CO_2 incubator. A4 cells (an epithelial ovarian cancer cell line developed earlier in our lab [3]) were used as the parental cell line for studies. Stable transfectants of A4 cells overexpressing Snail and Slug (SNA and SLA, respectively), radioresistant A4 cells (AR), paclitaxel-resistant A4 cells (AC), and transient Snail and Slug knockdown (siRNA Smartpool for Snail and Slug; procured from Dharmacon Inc., Chicago, http://www.dharmacon.com) were developed using standard procedures. SNA, SLA, AR, and AC demonstrated a typical fibroblast-like appearance characteristic of EMT compared with the parental epithelial A4 cells (supporting information Figs. 4B, 6A).

Data Mining from Gene Expression Databases

Gene expression dataset analyses relating to Snail and Slug available in the public domain (Gene Expression Omnibus [23]) and published literature [15, 24-26] were meticulously scanned to identify genes possibly regulated by these two TFs. This effort generated a subset of 340 genes (supporting information Table 3) that are differentially modulated by Snail and Slug, although the nature of their interactions—whether through direct promoter binding or systems networks involving other components—is not known. Further overlapping the three gene lists generated a small subset of 64 E-box-containing promoters for wet-lab validation (supporting information Table 3, marked in red).

RNA Extraction and Reverse-Transcriptase PCR

Total RNA was extracted from cells using Trizol—2 μg of which was reverse-transcribed into cDNA, and amplified for 30 cycles. Each cycle comprised 45-second denaturation at 94°C, 45-second annealing at the appropriate temperature midpoint standardized for each primer set, and 45-second extension at 72°C. β-actin was used as internal control in all reactions. Amplification was quantified by running amplicons on a 1.5% agarose gel and measuring band intensities as described above.

Immunoblotting

Total cellular protein was extracted in RIPA lysis buffer and estimated using DC protein estimation kit (Bio-Rad, Hercules, CA, http://www.bio-rad.com). Protein was denatured by heating at 95°C for 2 minutes, resolved on 10%–15% denaturing SDS-polyacrylamide gel electrophoresis, transferred onto a Hybond nitrocellulose membrane (semidry transfer; 20 V for 45 minutes),

blocked, and incubated overnight with primary antibody. After three buffer washes, the blot was exposed to horseradish peroxidase-conjugated secondary antibody for 3 hours, washed, and developed using Pierce SuperSignal West Pico chemiluminescence substrate (Pierce, Rockford, IL, http://www.piercenet.com).

Immunostaining for TUNEL

Coverslip-grown cells were fixed in 4% paraformaldehyde, permeabilized (0.1% Triton X-100 in 0.1% sodium citrate made in phosphate-buffered saline), and incubated either with or without dTT (dithiotreitol) enzyme containing terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling (TUNEL) mixture (Roche Diagnostics GmbH, Mannheim, Germany, http://roche-applied-science.com) at 37°C for 1 hour. Analysis for apoptosis was determined by images captured on a confocal microscope (Carl Zeiss, Jena, Germany, http://www.zeiss.com).

Flow Cytometry Cell Analysis

For cell cycle analysis, cells were harvested, fixed with chilled 70% ethanol for 15 minutes, washed in buffer, and stained with 50 μg/ml propidium iodide and 1 mg/ml RNase (Type IIA; Sigma-Aldrich, St. Louis, http://www.sigmaaldrich.com) for 30 minutes at 37°C. For TUNEL assay, cells were fixed in 4% paraformaldehyde and permeabilized as above, incubated either with or without dTT enzyme containing TUNEL Mixture (Roche Biosciences) at 37°C for 1 hour. Events were acquired on BD FACSCaliber (Becton, Dickinson and Company, Franklin Lakes, NJ, http://www.bd.com) and analyses carried out using Cellquest Software (Pro-Beckman Coulter, Krefeld, Germany, http://www.beckman.com).

Clonogenicity Assay (Soft Agar Assay)

In vitro tumorigenecity and anchorage-independent growth were assayed using standard methods. Cells were harvested and suspended in culture medium. One percent agarose was coated onto 35-mm plates, and further overlaid with cell suspension (5,000 cells in 0.5% agarose). Plates were incubated in humidified tissue culture incubator at −37°C, 5% CO_2, for 3 weeks. Visible colonies were counted under a phase-contrast microscope.

Statistical Analysis

Unless mentioned otherwise, all experiments were done in triplicates and data are represented as mean ± SE. Paired *t* test was performed (SigmaStat software; Aspire Software International, Ashburn, VA, http://www.aspiresoftwareintl.com) to determine significant differences between the groups.

RESULTS

Genome-Wide Analysis of Snail and Slug Binding Sites

The probabilistic frequency of the Snail and Slug consensus binding site known as E-box (5′-CANNTG-3′) is 1 in 2,048. With the human genome being ~3.2 billion base pairs [27], several genes are expected to have an E-box in their promoter region. Our in silico analyses for comprehensive genome-wide identification of E-boxes listed 14,532 genes as having an E-box in their promoters (Fig. 1A; supporting information text; supporting information Table 1). The chromosome-wise distribution of these genes (Fig. 1B) indicated that promoters with two or three E-boxes are more frequent; few promoters are associated with more than five E-boxes (a maximal number of 64 E-boxes was found in *Grk1* promoter), whereas some chromosomes had several promoter regions lacking E-boxes (~25% genes in chromosomes 1, 7, 9, 10, 11, 18, and X). This range of frequency distribution of E-boxes, from a total absence to very large numbers, suggests that E-box occurrence is not entirely random in the genome.

To filter this list of gene targets using Snail and/or Slug specificity in the above identification scheme, we carried out ChIP-on-chip to further screen for targets in an epithelial ovarian cancer cell line A4 (established earlier in our lab from tumor ascites of a patient with serous epithelial ovarian carcinoma [3]). Based on a minimum of twofold increased intensity over input DNA, 3,522 genes (Fig. 1C; supporting information Table 2) were identified to be direct targets of either Snail (10.82% targets), Slug (38.19%), or both (13.04%). Functional analyses of these targets indicated that cellular processes of development, metabolism, chromatin modification, cell signaling, and cell adhesion were regulated through common targets; genes involved in chromatin remodeling were targeted by Snail and Slug on an individual level, whereas Slug alone appears to be involved in the regulation of cell cycle and metastasis. Despite this incremental understanding, we realized that the list thus generated could not be considered a true representation of the complete target repertoire, since some of the genes classically regulated by these TFs, including *CDH1*, *CLDN4*, *ITGB1*, and so on, were markedly absent. This inconsistency was attributed to the absence of E-boxes in the probes used on the commercial microarrays.

Guided by a motive of extracting tissue- and disease-specific gene expression patterns implied to be regulated by Snail and Slug toward a specific biological function, namely radioresistance or chemoresistance in ovarian cancer, we further undertook extensive mining of gene expression analyses data relating to these two TFs from public databases and published literature. This effort generated a second list of 340 genes (supporting information Table 3), some of which were further eliminated based on an absence of E-boxes in their promoters or upregulated expression levels. The latter is suggested to indicate either an indirect derepression effect or direct induction through an unknown mechanism [28]. Overlapping the three gene lists identified in the three methodologies described finally yielded a small subset of 64 E-box-containing gene targets (supporting information Table 3, marked in red) for wet-lab validation in ovarian cancer.

Differential Affinities of Snail and Slug in Binding to Certain Gene Promoters and Their Dynamic Modulation in Steady-State and γ-Irradiated Ovarian Cancer Cells

ChIP-PCRs using Snail or Slug antibodies to immunoprecipitate chromatin and specific primers designed to amplify E-boxes in the promoters of the 64 putative targets in the epithelial ovarian cancer cell line (A4) revealed a common set of 37 gene targets for the two TFs. Although the binding affinities were comparable for 12 gene promoters including the known classic targets of these genes, namely *CDH1*, *CLDN4*, *OCLDN*, *KRT18*, and so on (Fig. 2A), the individual targeting efficiencies of the two TFs varied for the remaining genes. A novel finding revealed through statistical analysis of their differential affinities (ratio of Snail/Slug binding) is the increased affinity of Snail for *TP53* and *BRCA2*, whereas Slug expressed a preferential binding to *ITGB1*, *CAV1*, *MUC1*, and *HOOK1* promoters.

We further probed whether the same target profile would be retained or altered in these cells under environmental stress such as exposure to γ-irradiation (LD50 dose was established as 20 Gy for 36 hours; supporting information Fig. 2A, 2B). Using ChIP-PCR assays, a total of 39 targets were identified in the irradiated A4 cells (A4i) that were common to both TFs (Fig. 2B; supporting information Fig. 5A; supporting information

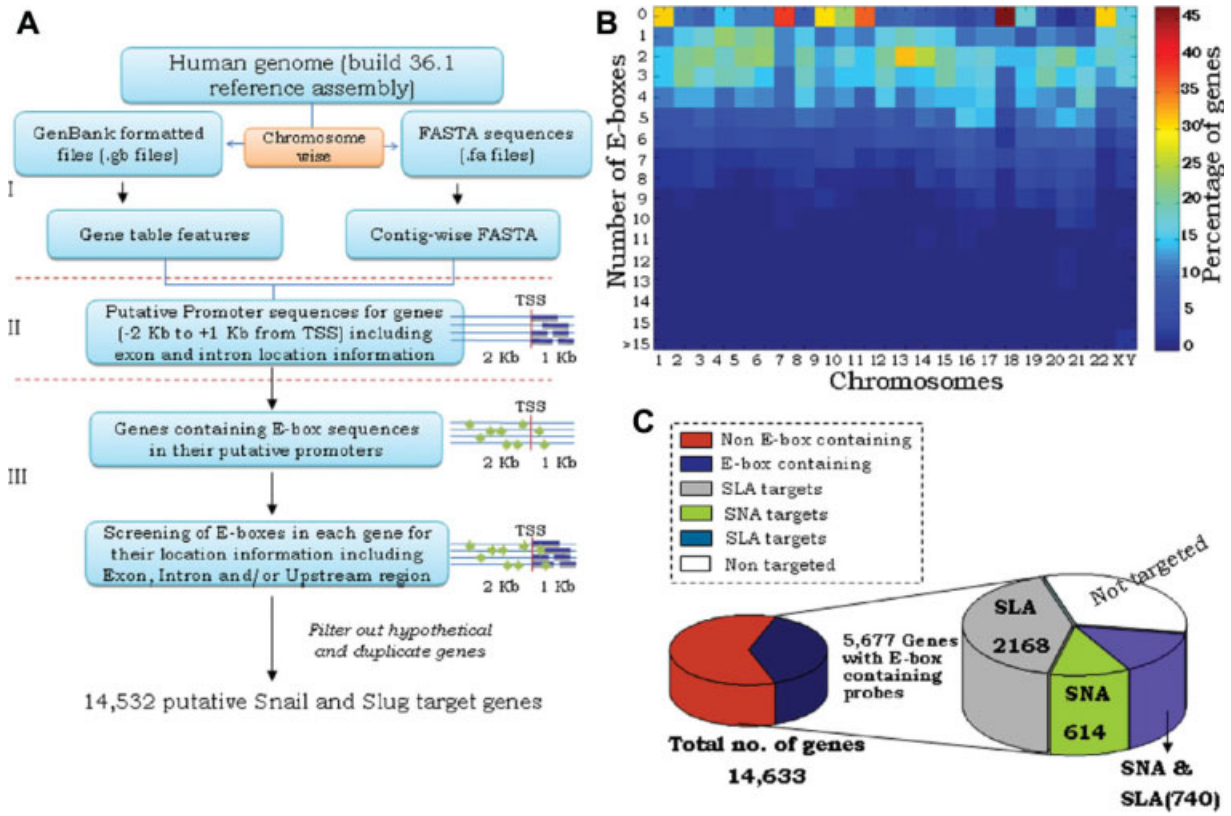

Figure 1. Genome-wide analysis of Snail and Slug binding sites in silico.**(A):** Outline of the computational approach used for genome-wide search of genes containing E-boxes in their putative promoter region. **(B):** Chromosome-wise distribution of genes with respect to the number of E-boxes in the putative promoter region represented as a pseudocolor plot. Chromosomes are represented on the *x*-axis; number of E-boxes, on the *y*-axis. The color of each cell indicates the percentage of genes containing E-boxes in putative promoter regions (−2 kb + 1 kb from TSS). **(C):** Summary of the genes enriched on the 244k promoter arrays by chromatin immunoprecipitation and promoter array analysis using Snail- or Slug-specific antibodies. Shown on the left is the distribution of E-box-containing and noncontaining genes; the pie on the right represents the distribution of Snail, Slug, and Snail-Slug common targets. Abbreviations: SLA, stable transfectants of A4 cells overexpressing Slug; SNA, stable transfectants of A4 cells overexpressing Snail; TSS, transcription start site.

Table 4). A majority of the 20 targets that were retained from steady-state A4 cells had increased binding affinity (*CDH1*, *CLDN4*, *KRT18*, *CAV1*, *BRCA2*, *MMP9*, *SNAI1*, *SNAI2*, and *TP53*); 19 genes emerged as novel targets in the irradiated state that could be significant either in cell survival (*BBC3*, *BCL2*, *EPHA2*, *BID*, *CDNK1A*) or in gene regulation (*SIN3A*, *MAPK3*, *CTNNA1*, *CTNNB1*); whereas 17 targets (*NANOG*, *HDAC1*, *HDAC3*, *CLDN3*, *OCLN*, *MUC1*, *MMP9*, etc.) were lost on irradiation (Fig. 2B; supporting information Fig. 5A). These results suggest a definitive modulation of the direct targets of these molecules under conditions of radiostress.

Profiling for Snail and Slug targets in ectopically overexpressing Snail and Slug A4 clones (SNA and SLA, respectively; supporting information text; supporting information Fig. 4A, 4B) identified a perfect overlap of 28 genes—a reduction in the number of targets from steady-state A4 cells (supporting information Fig. 5B). Twenty-five of these targets were retained from steady state—albeit with varying binding affinities; three novel targets (*MAPK3*, *BBC3*, *PKP2*) were gained (supporting information Table 3), whereas 12 targets at steady state, including *NANOG*, *HDAC1*, *HDAC3*, *MMP9*, and so on, known to contribute to tumorigenesis were lost (Fig. 2Ci). Exposing SNA and SLA cells to γ-irradiation (SNAi and SLAi cells, respectively) generated 16 novel targets including *CCND2*, *CDNK1A*, *BCL2*, *KLF4*, and so on (Fig. 2Cii; supporting information Fig. 5C), whereas 15 genes were retained from steady state (supporting information Table 4). A shift in binding preference was noted for the genes *BRCA2* and *RIN1* to Snail in SNAi cells after irradiation from earlier preferential binding to Slug in SLA cells (Fig. 2Cii; supporting information Fig. 5C). Binding to *HOOK1*, *KRT19*, *TP53*, and *PKP2* promoters was lost on γ-irradiation, possibly indicating derepression of these genes.

To identify target promoters bound at the natively elevated dosages of both TFs in the radioresistant AR cells (developed to resist up to 10 Gy γ-radiation; supporting information Fig. 3), ChIP-PCRs were carried out using Snail- or Slug-immunoprecipitated chromatin (ARSN and ARSL, respectively). Twenty-eight gene targets were thus identified—25 being retained from steady state (Fig. 2Di; supporting information Fig. 5D; supporting information Table 3); 3 novel (*BBC3*, *EPHA2*, and *ITGB1*) and 12 gene targets were lost including *NANOG*, *HDAC1*, *HDAC3*, *MMP9*, and so on, suggesting their derepression. Enhanced promoter binding of *Hook1* to Snail and *TP53*, *CRB3*, and *BRCA2* to Slug was evident in AR cells (supporting information Fig. 5D). Exposure of AR cells to γ-irradiation led to a further modulation of 28 targets (Fig. 2Dii; supporting information Table 4), with emergence of 11 novel targets (*BCL2*, *CASP9*, *CDNK1A*, *PCNA*, etc.) associated with cell survival (supporting information Fig. 5E). A few genes were retained from steady state, whereas seven genes, including *SMAD4*, *ITGB1*, *LAMA3*, *SPP1*, *TIMP3*, *CLDN7*, and *PLAU*, were lost on irradiation possibly indicating a derepression as above (Fig. 2Dii).

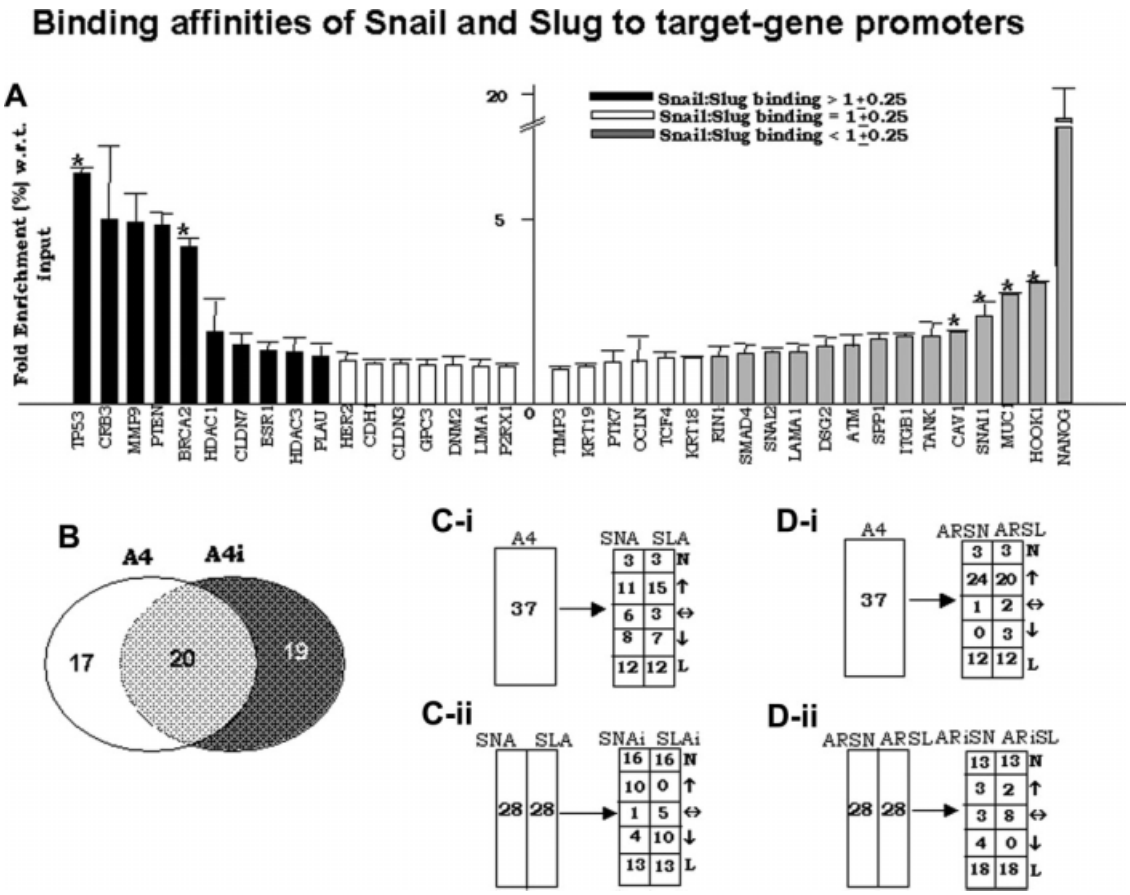

Figure 2. Modulation of gene targets by Snail and Slug in different cell systems. **(A):** Binding of Snail and Slug to target gene promoters in steady-state A4 cells detected by chromatin immunoprecipitation polymerase chain reaction (PCR) assay. Fold enrichment refers to ratio of PCR amplification (determined by densitometry) in Snail pull down versus Slug pull down for the same gene promoter. Each amplification value was initially normalized with that of the input sample; statistical significance was determined using paired *t* test (p value $< .05$). **(B):** Venn diagram showing novel, retained gene targets in γ-irradiated A4 over parental A4 cells. **(Ci):** Panel indicates the target modulation by Snail and Slug in SNA and SLA cells pulled down with Snail- or Slug-specific antibody, respectively, compared with steady-state A4 cells (total 37 targets). **(Cii):** Panel indicates the target modulation by Snail and Slug in SNA and SLA cells immunoprecipitated with Snail- or Slug-specific antibody, respectively, in postirradiated cells (SNAi and SLAi cells, respectively), compared with steady-state SNA and SLA cells (total 28 targets). **(Di):** Panel indicates the target modulation by Snail and Slug in AR cells immunoprecipitated with either Snail- or Slug-specific antibody, respectively (ARSN and ARSL cells, respectively), compared with steady-state A4 cells (total 37 targets). **(Dii):** Panel indicates the target modulation by Snail and Slug in ARSN or ARSL postirradiated cells (referred to as ARiSN and ARiSL cells, respectively), immunoprecipitated with Snail- and Slug-specific antibody, compared with steady-state ARSN/ARSL, respectively (total 28 targets). Denotations: number of targets that were novel (N), retained with enhanced (↑)/equal (↔)/reduced (↓) binding efficiencies, or lost (L). Abbreviations: ARiSL, ARSL postirradiated cells; ARiSN, ARSN postirradiated cells; ARSL, Slug-immunoprecipitated chromatin; ARSN, Snail-immunoprecipitated chromatin; SLA, stable transfectants of A4 cells overexpressing Slug; SLAi, SLA cells exposed to γ-irradiation; SNA, stable transfectants of A4 cells overexpressing Snail; SNAi, SNA cells exposed to γ-irradiation; w.r.t., with respect to.

Upregulation of Snail and Slug in Ovarian Cancer Cells Is Associated with Enhanced Cell Survival and Acquisition of Radioresistance

To test more directly and unambiguously for protective effects of these TFs in cancer cells, we used a comprehensive cell system composed of A4, SNA, SLA, and AR (supporting information Fig. 3). The latter were seen to acquire a native overexpression of Snail and Slug during the development of radioresistance (supporting information Fig. 4C–4E) that supported our hypothesis of a positive association of Snail and Slug with the resistant phenotype. Exposure of these four cell types to γ-irradiation elevated Snail and Slug mRNA and protein levels (Fig. 3A). The concurrent repression of the Snail and Slug target *CDH1* in all cell systems compared with the parental cells is indicative of the functional activity of these TFs.

Cell cycle and survival analyses of the above cell types indicated that A4, SNA, and SLA cells exhibited similar cell cycle kinetics, whereas AR cells had a distinctive profile, with a majority of cells being in G2/M phase and a small fraction of cells being aneuploid (Fig. 3B). On exposure to γ-radiation, a high fraction of all cells tend to accumulate in the G2/M phase. Strikingly, a third of the irradiated parental A4 accumulated in the sub-G0 phase (indicative of DNA fragmentation and apoptosis). Confocal images and flow cytometry data of cells assayed for TUNEL revealed that on exposure to γ-irradiation, a significantly high fraction of A4 cells, moderate numbers of SNA, and minimal fractions of SLA and AR populations were seen to be apoptotic (Fig. 3C, 3D) with respect to parental cells. Further profiling long-term functionality using in vitro soft agar colony-formation assays showed that, whereas at steady state elevated levels of Snail and Slug in the SLA, SNA, and AR

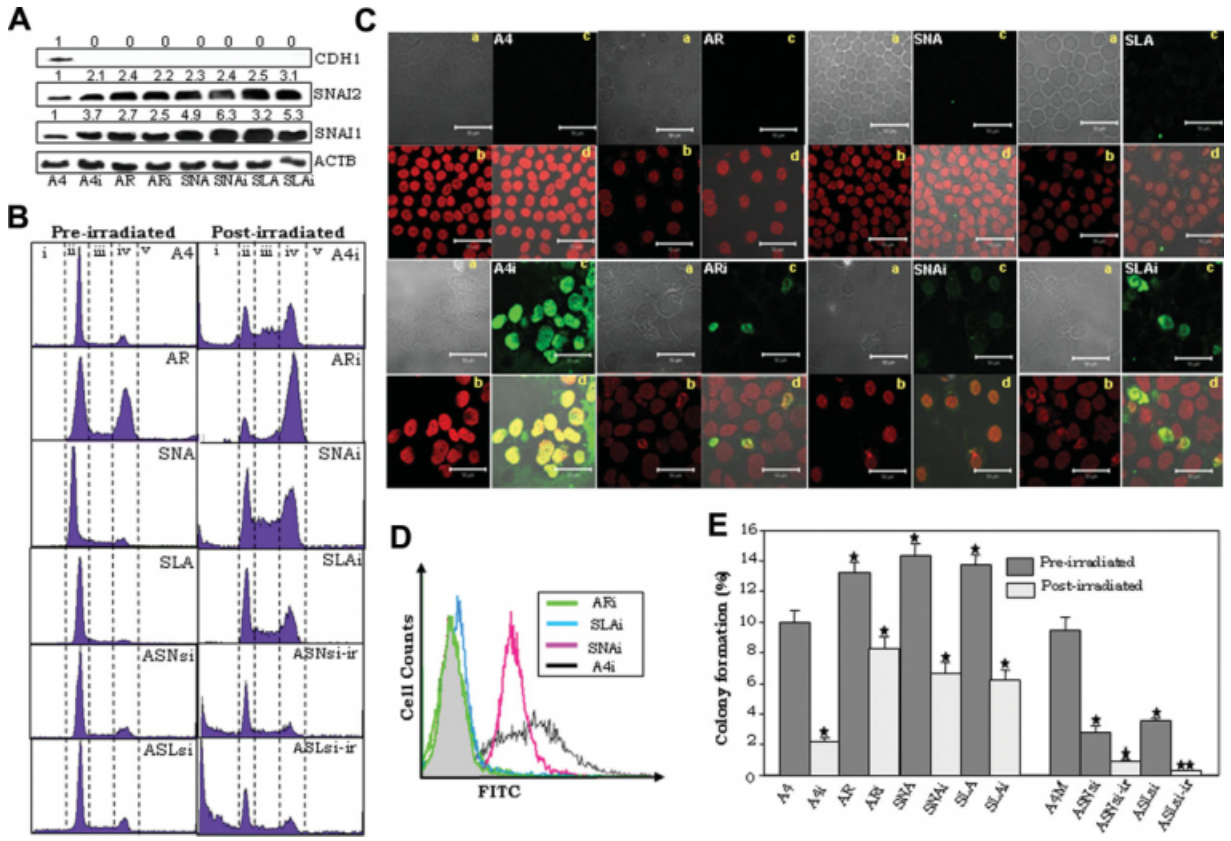

Figure 3. Radioprotective role of Snail and Slug against apoptosis. **(A):** Western blotting analysis of Snail, Slug, and E-cadherin (CDH1) expression in A4, AR, SNA, and SLA cells at steady state and on γ-irradiation (20 Gy for 36 hours: A4i, ARi, SNAi, and SLAi, respectively). ACTB was used as a loading control. Numbers on panel represent the ratio of densitometric value of each band normalized with reference to the loading control, compared with parental A4 control expression. **(B):** DNA content profiles captured through propidium iodide (PI) staining of A4, SNA, SLA, AR, ASNsi, and ASLsi cells at steady state and on γ-irradiation (20 Gy for 36 hours). In each histogram, panels **(i), (ii), (iii), (iv),** and **(v)** represent sub-G0, G0/G1, S, G2/M, and aneuploid cell populations, respectively. **(C, D):** Confocal images and plot overlay for detection of apoptotic cells by terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling (TUNEL) assays in A4, SNA, SLA, and AR cells at steady state and on γ-irradiation (20 Gy for 36 hours; panels **(i), (ii), (iii),** and **(iv)** represent TUNEL staining, phase contrast, nuclear staining by PI, and merged images, respectively). Scale bars represent 50μ. **(E):** In vitro clonogenicity assay of steady-state (A4, SNA, SLA, AR, ASNsi, and ASLsi) and postirradiated (A4i, SNAi, SLAi, ARi, ASNsi-ir, and ASLsi-ir) cells. Representative graph shown for experiments done in triplicate; $p \leq .05$. Abbreviations: A4i, A4 cells exposed to γ-irradiation; AR, radioresistant A4 cells; ARi, AR cells exposed to γ-irradiation; ASLsi, A4 cells transfected with Slug; ASLsi-ir, postirradiated ASLsi cells; ASNsi, A4 cells transfected with Snail; ASNsi-ir, postirradiated ASNsi cells; FITC, fluorescein isothiocyanate; SLA, stable transfectants of A4 cells overexpressing Slug; SLAi, SLA cells exposed to γ-irradiation; SNA, stable transfectants of A4 cells overexpressing Snail; SNAi, SNA cells exposed to γ-irradiation.

cells correlated with an increased clonogenicity compared with parental A4 cells, exposure to irradiation drastically decreased this potential in A4 cells, although SNA, SLA, and AR cells continue to form colonies in soft agar, indicating a capacity for re-entry into cell cycle, cell survival, proliferation, and retention of tumorigenic capabilities (Fig. 3E).

We then sought to determine whether decreased levels of these TFs would reverse the above effects. Knocking out individual expression by transfecting parental A4 cells with either Snail or Slug siRNA (to generate ASNsi and ASLsi cells, respectively) indicated a distinct shift of the G0/G1 cell population toward sub-G0 phase in postirradiated ASNsi as well as ASLsi cells (ASNsi-ir and ASLsi-ir cells, respectively; Fig. 3B). This sub-G0 fraction was higher in Slug knockdown (50%) than in Snail knockdown (30%) cells, hinting possibly at a more protective role of Slug. Clonogenicity assays further indicated that silencing of either TF resulted in lower colony-formation efficiencies than that of scrambled sequence transfected A4 cells; furthermore, this capacity was almost completely abrogated after irradiation when either TF was silenced (Fig. 3E). Together our observations support a definitive correlation of the two TFs with radioresistance, since cells that express elevated levels of Snail and Slug are more capable of overcoming cell cycle arrest at the G2/M checkpoint to continue cycling, thereby effecting cell survival.

Chemoresistance of Ovarian Cancer Cells Is Also Associated with Upregulated Snail and Slug Levels

Toward exploring the possible involvement of Snail and Slug in chemoresistance—a serious concern in ovarian cancer, we developed A4 cells resistant to 5 nM paclitaxel (AC cells). These cells are spindle shaped and, like the AR clones, acquire a native overexpression of the two TFs during development of chemoresistance (supporting information Figs. 3, 6A). On exposure of parental A4, AC, SNA, and SLA to the LD50 dose of paclitaxel (0.7 nM, supporting information Fig. 2C; supporting information text), increased Snail and Slug mRNA and protein levels were evident in the treated cells (A4-px) compared with A4 and SNA, but not to the same extent in the treated AC, SLA, and SNA cells (AC-px, SLA-px, and SNA-px, respectively; supporting information Fig.

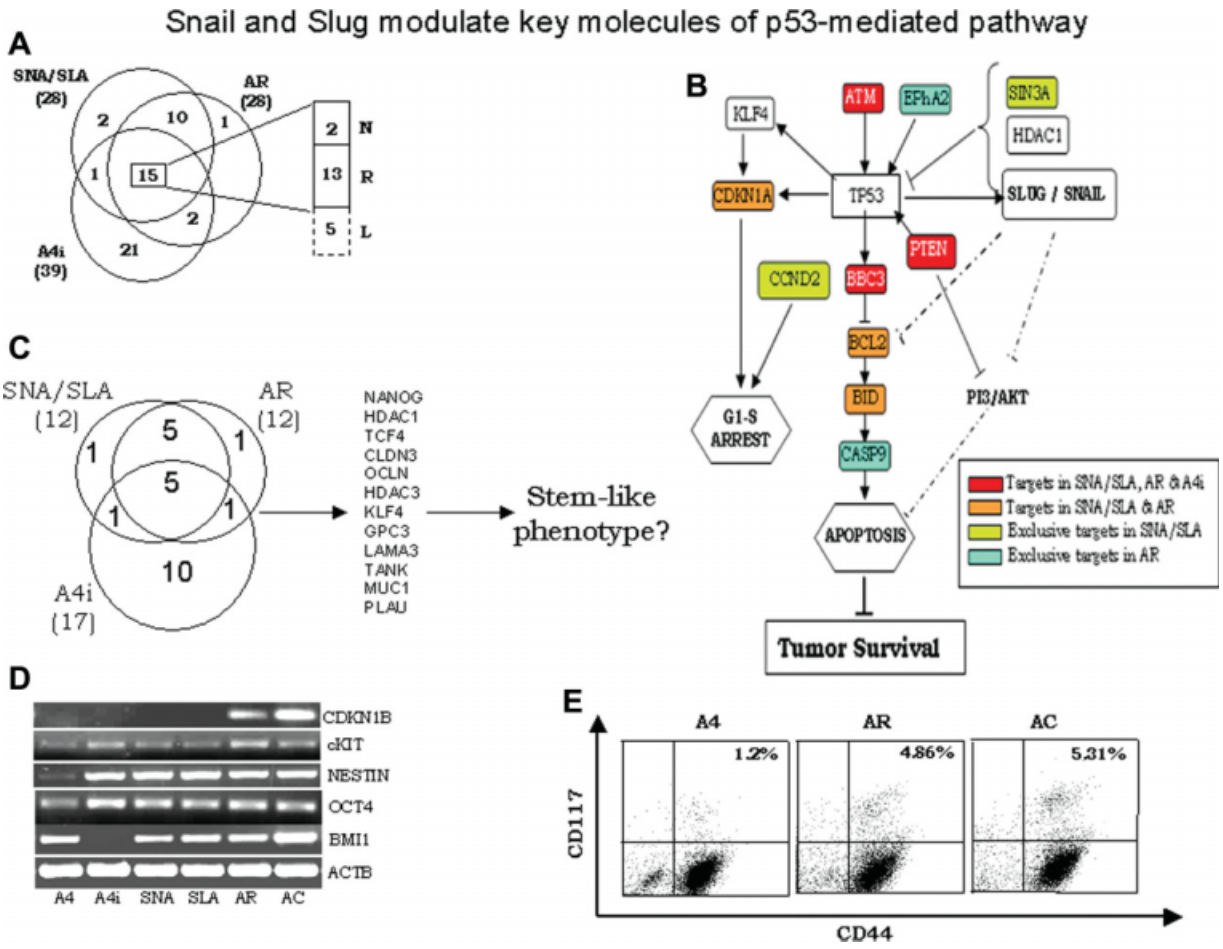

Figure 4. Ectopic or natively upregulated levels of Snail and Slug repress key molecules in p53-mediated apoptosis. **(A):** Venn diagram representing the overlap of gene targets in SNA/SLA (ectopic) and A4i, AR (native) cells upregulating Snail and Slug. Intercepting values indicate the common genes between the respective cell types. Of the 15 genes that were common to all cells, 2 were novel (N), 13 were retained (R), and 5 were lost (L) compared with the parental A4 cells. **(B):** Schematic representation indicating genes in p53-mediated DNA damage response pathway targeted by Snail and Slug in our study. **(C):** Venn diagram representing the overlap of gene targets that are lost in SNA/SLA (ectopic) and A4i and AR (native) cells upregulating Snail and Slug compared with parental A4 cells. Intercepting areas indicate numbers of common genes lost as targets—also included in the list. **(D):** Expression levels of stem cell markers in A4i, SNA, SLA, AR, and AC cells having enhanced expression of Snail and Slug compared with parental A4 cells. ACTB was used as an internal control. **(E):** Representative dot blot of CD44 and CD117 (c-kit) expression in AR and AC cells compared with parental A4 cells. Abbreviations: A4i, A4 cells exposed to γ-irradiation; AC, paclitaxel-resistant A4 cells; AR, radioresistant A4 cells; L, lost; N, novel; R, retained; SLA, stable transfectants of A4 cells overexpressing Slug; SNA, stable transfectants of A4 cells overexpressing Snail.

6B, 6C), which already express higher levels at steady state. Cell cycle analyses of these cells indicated significant apoptosis and G2-M arrest in A4-Px cells compared with parental A4 cells. However cell cycle profiles were not altered to the same extent in AC cells (supporting information Fig. 6D). Annexin V staining of these two cell types further confirmed the above findings (supporting information Fig. 6E).

Ectopic Overexpression and Native Upregulation of Snail and Slug Affects Specific Pathways

A striking observation was of the similarity between cellular states wherein Snail and Slug are upregulated vis-à-vis ectopic expression (SNA/SLA cells) and native upregulation (irradiated [A4i] and radioresistant [AR] cells), with respect to new targets acquired, retained, or lost over parental A4 cells. Moreover, the degree of this overlap was higher between the SNA, SLA, and AR cells than with the A4i cells; this correlates with higher postirradiation survival in the former three cell types as described earlier (Fig. 3). From the genes modulated in these states, we derived a consolidated overlap of 15 gene targets (Fig. 4A), of which two were novel targets (*BBC3*/*PUMA* and *MAPK3*), whereas 13 were retained from the parental A4 cells (*ATM*, *BRCA2*, *CDH1*, *PTEN*, etc.; supporting information Table 5). The 15 genes repressed by Snail and Slug under stress are suggested to be involved in mediating inhibition of either p53, p53 feedback loop 2, or EGF receptor signaling pathways (identified through pathway analyses using the gene expression analysis tool PANTHER; supporting information Fig. 7). Three of these 15 genes, namely ATM, *PTEN*, and *BBC3*/*PUMA*, were components of the p53 feedback loop 2; additional components of p53 signaling include *KLF4*, *CDKN1A*, *BCL2*, *BID*, *EPHA2*, and *CASP9* in SNA/SLA and/or AR cells (supporting information Fig. 8). The repression was validated and a good correlation between promoter occupancy of *BBC3*, *EPHA2*, and *BCL2* by Snail/Slug with their repression at the mRNA and protein levels was observed (supporting information Fig. 8); whereas similar promoter occupancy of *PTEN*, *ATM*, *CCND2*, *SIN3A*, and *CASP9* correlated with gene repression at the transcript level

(supporting information Fig. 9A). Thus, repression of various components of the p53 pathway by Snail and Slug under conditions of stress may mediate resistance to apoptosis.

Gene Targets Lost Through Natively Upregulated Snail and Slug Levels May Influence "Stemness"

NANOG, *HDAC1*, *CLDN3*, *OCLDN*, and *TCF4* were identified as possibly being derepressed through reduced promoter occupancy of Snail and Slug in cells that upregulate level(s) of these TFs, namely A4i/SNA/SLA/AR cells (supporting information Table 5). Additionally, *HDAC3*, *KLF4*, *GPC3*, *LAMA3*, *TANK*, *MUC1*, and *PLAU* were also lost as targets in at least two of these cell types (Fig. 4C; supporting information Table 6). We reasoned that such a derepression could indicate a definitive advantage for a cancer cell under stress. By virtue of its being a master transcriptional regulator for maintenance of undifferentiated stem cells [29], derepression of *NANOG*, *HDAC1*, and *HDAC3*, complemented by re-expression of KLF4 (associated with acquisition of pluripotency), suggests a mechanism for the acquisition of stem-like characteristics by these cells toward resisting genotoxicity. Good correlation of loss in promoter occupancy was evident with the re-expression of *NANOG*, *HDAC1*, *HDAC3*, *KLF4*, *CLDN3*, and *TCF4* at the transcript level (supporting information Fig. 9B). As further confirmation of association of derepression of a selective subset of genes with acquisition of stem-like characteristics, we probed A4, A4i, SNA, SLA, AR, and AC cells for the expression of additional stem cell markers. This profiling revealed a distinct upregulation of OCT4, NESTIN, and c-KIT (CD117); BMI-1, however, was markedly absent in the A4i cells (which have low levels of Nanog expression and cell survival); whereas the quiescence marker *CDKN1B* was expressed only in the AR and AC cells (Fig. 4D). The differential effects may be attributed to achievement of optimal dosage and cooperative effects of both TFs through a native upregulation during acquisition of radioresistance or chemoresistance. We further probed the three cell types, namely parental A4, AR, and AC cells, for expression of the surface phenotype $CD44^{+}/CD117^{+}$ that has been recently reported for ovarian cancer stem cells [30]. A fourfold to fivefold increase in the putative stem cell populations was evident in both the resistant cell types (Fig. 4E). The derepression of key molecules by Snail and Slug on exposure to stress, and their contribution to acquisition of resistance, thus appears to be one of the key contributory mechanisms in ovarian cancer resistance.

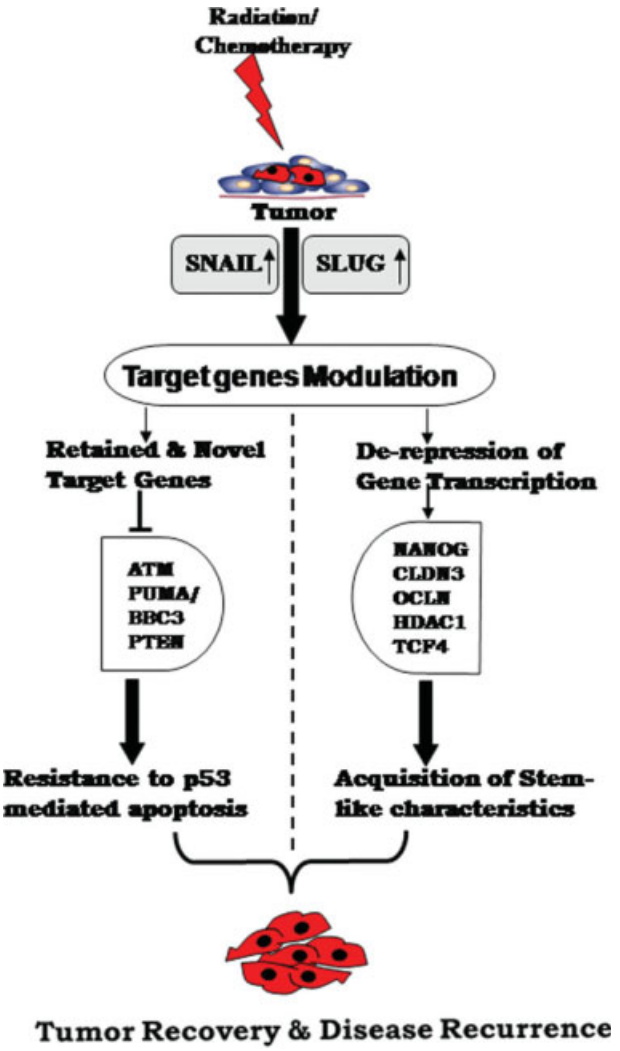

Figure 5. Model summarizing the mechanism of action of Snail and Slug in ovarian cancer cells exposed to stress. Snail and Slug expression is trigged in response to radiation and chemotherapy. These molecules perform two diverse effects, namely (a) repression of genes involved in p53-mediated apoptosis, which leads to enhanced cell survival, and (b) derepression of self-renewal genes, which leads to the acquisition of a stem cell-like phenotype. The outcome of these effects is failure of therapy, tumor cell recovery, and disease recurrence.

Discussion

In the present study, we report the upregulation of the transcriptional repressors Snail and Slug under conditions of radio-/drug-induced stress to modulate at least 47 genes in ovarian cancer (supporting information Fig. 10). Although this number of genes is likely to be a gross underrepresentation of the total repertoire of Snail and Slug targets, it extends the current understanding of their biological functionality in cancer. Through identification of this definitive modulation, we realized that Snail and Slug regulate at least two programs besides EMT in a cancer cell (Fig. 5):

(a). Direct participation of Snail and Slug in p53-mediated pro-survival signaling through active repression of pro-apoptotic genes *PUMA*/*BBC3*, *ATM* and *PTEN*. The transcription factor p53, activated by cellular stress, is reported to involve transactivation of Slug for partnering in its decision to arrest, repair, and mediate cell survival under stress [31, 32]. Slug represses *PUMA*/*BBC3*, a potent proapoptotic molecule [33], and is associated with radiosensitivity of stem cells [10, 11, 34, 35] and cisplatin sensitivity in ovarian cancer cells [36]. The role of Snail and Slug identified in the present study provides a mechanistic understanding of repression of *PUMA* that, although being in concordance with previous reports of Slug-mediated radioresistance of hematopoietic stem cells, appears to additionally involve inactivation of other components of the p53-mediated apoptotic pathway in the context of cancer including *ATM* and *Pten*.

(b). Snail and Slug regulate indirect/passive activation of a self-renewal program through loss of binding to specific gene promoters including *NANOG*, *HDAC1*, *TCF4*, *KLF4*, *HDAC3*, *GPC3*, and so on that were earlier repressed under steady-state conditions. On examining this gene modulation, we realized that the stemness program thus

activated by Snail and Slug further involves expression of other "stem cell markers," including Oct4, Bmi1, and nestin, and a fourfold/fivefold increase in the number of $CD44^{+}CD117^{+}$ cells (representative of ovarian CSCs [30]). The derepression is strongly suggestive of de novo generation of CSCs through dedifferentiation of cancer cells; it is also highly unlikely that indigenous CSCs would proliferate rapidly under conditions of stress to generate the enhanced numbers of resistant cells reported in minimal residual disease [37]. Therefore, our findings provide a mechanistic understanding for reports on enrichment of stem cells in tumors after therapy [38], as does the recent report linking EMT with generation of CSCs [5].

It has been proposed that only a subset of primary tumor cells with a distinct immunophenotype that involves several stem cell markers (e.g., expression of homing molecules: CXCR4 [39], aldehyde dehydrogenase [40], etc.) has the capability to metastasize [41]. Our findings extend this understanding to suggest that stem-like cells generated through activation of Snail and Slug, and bestowed with three critical capabilities, namely EMT, resistance to p53-mediated apoptosis, and a self-renewal program, actually define the functionality and survival of metastatic CSCs. EMT provides a mechanism of escape to a new, less adverse niche; resistance to apoptosis ensures cell survival in conditions of stress in the primary tumor; whereas acquisition of stemness characteristics ensures generation of the critical tumor mass required for progression of micrometastases to macrometastases.

Conclusions

The term "stem cell" was coined in the context of colony-forming cells surviving radiation and repopulating the spleen [42]; this defining capability to bide environmental stress is a necessity in the normal niches wherein stem cells perform tissue regenerative functions throughout the life span of an organism. Identification of stem cells in cancer was immediately realized to have a more important repercussion vis-à-vis recalcitrance of tumors to therapy and occurrence of minimal residual disease [36, 37, 43]. However, the mechanism underlying the enrichment of stem-like cells after treatment has never been resolved.

In the present work, we report a probable model of the involvement of transcription factors Snail and Slug and an elegant modulation of their targets under conditions of stress that effectively mediates cell survival and involves the acquisition of stem-like characteristics to ovarian cancer cells. This identification not only provides mechanistic understanding of the enrichment of stem cells in posttreatment tumors, but furthermore, our data hint that the de novo generation of CSCs by Snail and Slug probably represents directed dedifferentiation of cells in tumors since it is highly unlikely that CSCs would continue to self-renew/proliferate under conditions of stress to generate the enhanced numbers of resistant cells. Although the precise molecular intricacies of these effects in a recalcitrant tumor could be more complex and involve cooperative functioning of other molecules and could partly be related to continued evolution of resistance [44], our findings definitely suggest that Snail and Slug are critical determinants of disease progression. Although we have focused on these phenomena in ovarian cancer, our findings are possibly extendable to other cancers to represent a common, yet underestimated, mechanism that could make cancer cells difficult to eradicate.

Acknowledgments

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Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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