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NF- κ B promotes ovarian tumorigenesis via classical pathways that support proliferative cancer cells and alternative pathways that support ALDH⁺ cancer stem-like cells

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Abstract

Understanding the mechanisms supporting tumor-initiating cells (TIC) is vital to combat advanced stage recurrent cancers. Here we show that in advanced ovarian cancers NF- κ B signaling via the RelB transcription factor supports TIC populations by directly regulating the cancer stem-like associated enzyme aldehyde dehydrogenase (ALDH). Loss of RelB significantly inhibited spheroid formation, ALDH expression and activity, chemoresistance, and tumorigenesis in subcutaneous and intrabursal mouse xenograft models of human ovarian cancer. RelB also affected expression of the ALDH gene ALDH1A2. Interestingly, classical NF- κ B signaling through the RelA transcription factor was equally important for tumorigenesis in the intrabursal model, but had no effect on ALDH. In this case, classical signaling via RelA was essential for proliferating cells, whereas the alternative signaling pathway was not. Our results show how NF- κ B sustains diverse cancer phenotypes via distinct classical and alternative signaling pathways, with implications for improved understanding of disease recurrence and therapeutic response.

Keywords

tumor-initiating cells; NF- κ B; ovarian cancer; ALDH; CD133

INTRODUCTION

Ovarian cancer is the most lethal gynecological malignancy in the USA, resulting in over 15,000 deaths annually (1). Although most patients initially respond to platinum-based chemotherapy, over 70% of advanced stage ovarian cancer relapses within 24 months (2).

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Disease recurrence is a major cause of morbidity and mortality, and mechanisms of tumor relapse and chemoresistance are unclear. Evidence suggests tumor-initiating cells (TICs) drive tumorigenesis and regulate chemoresistance and tumor reformation following first-line therapy. More recently attention is drawn to understanding the dynamic collaborations of subpopulations of cells, including TICs, that support tumor heterogeneity, therapy resistance, and disease progression (3–5).

We previously demonstrated that specific components of classical NF- κ B signaling are required for aggressive tumor behavior and metastatic potential in subsets of ovarian cancers (6–8). NF- κ B signaling is a complex network that follows both *classical* (canonical) and *alternative* (non-canonical) cascades, defined by upstream kinases and ultimate activation of either p65/p50 (classical) or RelB/p52 (alternative) transcription factors. Elevated *classical* NF- κ B signaling has been observed in TICs of prostate, breast, and ovarian tumors (9–11), however the role of *alternative* NF- κ B signaling is not established. Both cascades are required for maintenance and promotion of breast cancer TICs (12). Classical and alternative NF- κ B pathways can regulate each other as well as integrate with other signaling pathways for fine-tuning functional outputs (13–16). NF- κ B target genes encode proteins and microRNAs important for the inflammatory response, cell survival, proliferation, adhesion, and interaction with the microenvironment. The diverse and complex roles of NF- κ B suggest this transcription factor family might regulate distinct functions in different cell types within heterogeneous cell populations.

Here, we investigated the hypothesis that classical and alternative NF- κ B signaling support different subpopulations of ovarian cancer cells. Although both pathways are required for tumorigenesis, our data show that they maintain different cancer cell phenotypes. Classical NF- κ B supports proliferative cells, whereas alternative NF- κ B supports cells with low proliferative potential bearing TIC markers. This study reveals a novel role for RelB in ovarian cancer and demonstrates mechanistically that NF- κ B can promote heterogeneity for efficient tumorigenesis and drug resistance. This novel insight into mechanisms of tumor heterogeneity in ovarian cancer will advance therapeutic approaches to improve clinical outcomes.

MATERIALS AND METHODS

Cell Lines and Culture Conditions

Characteristics of cell lines used in this study are provided in Supplemental Table 1. OVCAR8 (HTB-161), CAOV3 (HTB-75) and OV90 (CRL-11732) were obtained directly from American Type Culture Collection (ATCC) and maintained in Roswell Park Memorial Institute (RPMI) medium supplemented with 10% heat inactivated fetal bovine serum (FBS), 2 mM L-glutamine and penicillin-streptomycin (Gibco, Grand Island, NY) at 37 °C and 5% CO₂. OVCAR5 was obtained from NCI-Frederick DCTD tumor/cell line repository and maintained in RPMI with 10% FBS. ACI-23 cell line was provided by Dr. John Risinger and Memorial Health University Medical Center, Inc. and maintained in Dulbecco's Modified Eagle Nutrient Mixture F-12 (DMEM/F12) medium (Gibco) supplemented with 10% FBS, 2 mM L-glutamine and penicillin-streptomycin. Cell lines were authenticated via Short Tandem Repeat analysis by the Protein Expression Laboratory, Leidos, Frederick

National Laboratory, and used for study within 6 months of receipt. De-identified ascites specimens were collected as pathological waste from ovarian cancer patients and were determined to be outside of federal regulations for the protection of human subjects [45 CFR 46 (OHSRP #12727 and #12797)]. Viable tumor cells from ascites (VTCE, TGS1, TGS4) were purified using Ficoll separation and maintained in the same manner as ACI-23 cells. TIC-enriching conditions were created by culturing ovarian cancer cells in ultra-low attachment flasks (Corning) with stem cell media: DMEM:F12 (+ L-glutamine, + 15 mM HEPES) (Gibco) supplemented with 1% penicillin/streptomycin, 1% KnockOut serum replacement (Gibco), 0.4% bovine serum albumin (Sigma-Aldrich), and 0.1% insulin-transferrin-selenium (Gibco). TIC-enriching cultures were supplemented with fresh human recombinant epidermal growth factor (EGF) and basic fibroblast growth factor (FGF) every 2–3 days for a final concentration of 20 ng/ml and 10 ng/ml, respectively.

***In vivo* studies**

All animal studies were approved by the NCI Animal Care and Use Committee. All mice were fed doxycycline chow (200 mg/kg) for the duration of the study. OV90 and ACI23 cells were maintained in TIC-enriching conditions for one week prior to injections. For intraperitoneal (IP) xenografts, 1×10^6 OV90 cells in a volume of 500 μ l PBS were injected into the abdominal cavity of 8-week-old female athymic Nu/Nu mice. Tumor burden for mice injected IP was assessed by pathology after necropsy and collection of organs. For subcutaneous xenografts, 1×10^6 ACI23 cells in 500 μ l were subcutaneously injected into the right flank of 8-week-old female athymic Nu/Nu mice. For controls, 500 μ l PBS was injected into the left flank of each mouse. Mice were weighed and tumors measured using calipers twice weekly. Mice were sacrificed 24 days post inoculation. Single-cell suspensions were prepared from tumors using the Human Tumor Dissociation Kit and gentleMACS Dissociator (Miltenyi Biotec, Inc.). For bursa xenografts, 2×10^4 OV90 cells in 5 μ l were injected into the right bursa of 8-week-old female athymic Nu/Nu mice. For controls, 5 μ l PBS was injected into the left bursa of each mouse. Mice were sacrificed 65 days post inoculation. Ovaries were removed, weighed and fixed in 4% paraformaldehyde. Sections of liver, pancreas, peritoneal wall, and diaphragm were removed and fixed in 4% paraformaldehyde to assess metastatic spread. Organ sections were stained with Hematoxylin and eosin (H&E) and analyzed by a pathologist.

RNA Extraction and Quantitative real-time PCR (qRT-PCR)

Total RNA was isolated and purified using the RNeasy Mini Kit (Qiagen) as per manufacturer's protocol and treated with DNase. Final RNA concentration was determined with NanoDrop spectrophotometer using the 260/280 absorbance ratio. Total purified RNA was reverse transcribed with random primers using Taqman reagents (Applied Biosystems). Resulting cDNA was used as a template for qRT-PCR. Analysis of gene expression was performed on ViiA7 Real-time PCR system (Applied Biosystems) using Taqman probe assays and GAPDH as control. Quantitation and normalization of relative gene expression were accomplished using comparative threshold cycle method or C_T . Catalog numbers for commercial primers are provided in Supplemental Table 2.

Gene Expression

Total RNA was isolated from 3 independent cultures of ovarian cancer cells grown on traditional or ultra low attachment 75 cm cell culture flasks in presence of traditional serum-containing media or serum-free stem cell media. Samples were submitted to Laboratory of Molecular Technology, SAIC/NCI-Frederick and RNA quality checked on an Agilent Bioanalyzer; only samples having high quality score (RIN>9) were used for microarray analysis. RNA (1ug) was reverse transcribed with T7-oligo (dT) primers and labeled with biotin using the Affymetrix One Cycle Target Labeling Kit. All replicates of experimental samples were prepared, labeled and hybridized to Affymetrix H133 Plus 2.0 gene chips and scanned on an Affymetrix GeneChip scanner 3000 (Affymetrix, Santa Clara, CA). Data sets are available on the GEO repository (GSE94358).

Western blot

Whole cell protein was extracted from ovarian cancer cell lines using standard methods with NP-40 lysis buffer. BCA Protein Assay Kit (Thermo Scientific) was utilized to determine protein concentrations. 30 ug protein was loaded for each experiment. SDS-PAGE was performed using NuPage system (Invitrogen) and Luminata HRP Chemiluminescent Detection Reagents (Millipore). Antibodies were purchased from Cell Signaling Technologies (p-p65, 3033S; RelB, 4922S), Millipore (p52/100, 05-361; GAPDH, MAB374), Santa Cruz Biotechnology (p65, SC-372), Abcam (ALDH1, ab52492; ALDH1A2, ab96060), and Miltenyi Biotec (CD133, 130-092-395). Densitometry analysis was performed using ImageJ Software 1.48v.

Immunofluorescence

Ovarian cancer cells or spheroids from traditional adherent or TIC-enriching culture conditions, respectively, were collected after 5 days in culture. Cells were pelleted, washed with PBS, and fixed at room temperature using 4% paraformaldehyde and permeabilized with 0.3% Triton X-100. Cells were sequentially stained in following order and condition: ALDH1A1 or ALDH1A2 rabbit (Abcam) (1:100 overnight at 4 degrees), Alexafluor 594 monovalent Fab fragment goat anti-rabbit secondary (Jackson ImmunoResearch) (1:50 1hr at room temperature), Alexafluor 647 Ki67 rabbit antibody (BioLegend) (1:50 overnight at 4 degrees), Alexafluor 555 RelB rabbit antibody (Bioss) (1:100 1hr at room temperature), and Alexafluor 488 p65 rabbit antibody (Abcam) (1:100 1hr at room temperature). Cells were washed with PBS between each antibody staining. Finally, cells were stained with DAPI (Thermo Scientific) (1:1000) for 15 minutes at room temperature and mounted on slides using ProLong Diamond antifade mountant (Life Technologies). Images were acquired with a Zeiss LSM880 NLO laser scanning confocal microscope equipped with 34 channel spectral detector and 63x plan-apochromat (N.A. 1.4) oil immersion objective lens (Carl Zeiss Microscopy, LLC, Thornwood, NY). Image acquisition details can be found in Supplemental Experimental Procedures. Further quantification and statistical analysis of fluorescence levels in populations of individual cells was performed using custom-written software in Matlab (Mathworks).

RNA Interference

Silencing of RELA, RELB, GAPDH positive control, and Non-targeting negative control was achieved using SMARTchoice inducible lentiviral shRNAs (GE Dharmacon) designed with the human EF1 α promoter and red fluorescent protein (TurboRFP) reporter gene. OV90, ACI23 and VTCF003 cells were transduced according to manufacturer's protocol. Briefly, cells were transduced with viral particles in serum-free transduction media for 24 hours followed by selection with 2 μ g/ml puromycin for 7 days. For transient gene knockdown studies On-Target Plus Smartpool siRNAs were purchased from Dharmacon for ALDH1A1 (cat# L-008722-00), ALDH1A2 (Cat# L-008118-00) and non-targeting control (Cat# D-001810-10-20). Briefly, cells were seeded at 60% confluence and transfected with 20 nM siRNA for 24 hours before proceeding with functional assays.

Flow Cytometry

ALDH activity was evaluated using the Aldefluor Kit (StemCell Technologies) according to manufacturer's instructions. Following staining procedure for ALDH, cells were incubated with APC conjugated CD133 antibody (Miltenyi Biotec) 1:20 in the Aldefluor assay buffer for 30 minutes on ice protected from light. To evaluate proliferative populations cells were fixed and permeabilized as above and incubated with Alexafluor 647 Ki67 rabbit antibody (BioLegend) 1:100 for 30 minutes on ice. Flow cytometry data collection was performed on BD FACSCalibur or LSRFortessa using CellQuest Pro or FACSDiva acquisition software, respectively (Becton Dickinson). Data were analyzed using FlowJo software (Tree Star). Sorting of ALDH positive and negative cells was performed on a BD FACSARIA Fusion sorter.

Spheroid formation assay

To generate ACI23 and OV90 spheroids, 500 cells/well in 50 μ L were seeded in TIC-enriching conditions (stem cell media in ultra-low attachment flat bottom 96 well plates (Corning)). After 7 days in culture 3 fields per well were imaged at 10 $\times$ magnification using Axio Observer A1 Inverted Microscope and analyzed using ImageJ Software 1.48v. Spheroids with diameter $\geq$ 50 microns were quantified. Spheroid efficiency was calculated as spheroids $\geq$ 50 microns/cells per well).

Cell viability assay

Cell viability was assessed using CellTiter Glo luminescent reagent (Promega). 100 μ L of reagent was added to each well after analysis; 250 μ L of cells with luminescent reagent was transferred to a white, flat bottom 96 well-plate (Corning). SpectraMax M5 was utilized to quantify cell viability using SoftMax Pro Software 5.8.3.

Chromatin Immunoprecipitation-qPCR (ChIP-qPCR) assay

The SimpleChIP Enzymatic Chromatin IP Kit (magnetic beads) was purchased from Cell Signaling Technology (Danvers, MA) and assays performed according to the manufacturer's instructions. Antibodies for RelB were also from Cell Signaling (catalog #10544). Promo was used to evaluate DNA sequences for transcription factor binding sites (http://algggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3). The first 5,000

bases upstream of the transcription start site were screened for binding motifs that correspond to NF- κ B consensus binding sequence. The quantification of transcription factor binding to target genes was calculated by measuring the ratio of ChIP-to-Input and the normal rabbit IgG antibody served as a negative control. All primers used for ChIP-PCRs are listed in Supplemental Table 2.

Statistical Analysis

Raw microarray (CEL) data was processed using robust multi-array analysis (RMA) using the oligo package in Bioconductor (17). After quality control check, 2 outlier samples were removed, and differentially expressed genes were generated using Limma package taking in account batch effect (18,19). Other statistics were performed in Prism7 (GraphPad) using data acquired from at least three biological replicates as noted. Statistical analysis of immunofluorescence images was performed in Matlab (Mathworks). *P* values were calculated as described in figure legends.

RESULTS

We previously showed that ovarian cancer cells grown in TIC-enriching conditions form spheroid structures with high ALDH activity and are more tumorigenic than the same cells grown in traditional culture conditions (20). In this study we sought to identify gene expression changes incurred by cells grown in TIC-enriched conditions and to characterize the role of classical and/or alternative NF- κ B in supporting cells in TIC cultures.

Differential gene expression was determined by comparing profiles from cells grown in traditional adherent culture (A) with cells grown in TIC-enriching conditions (T). Genes were selected as differentially expressed based on cutoffs of fold change > 2.0 and *p*-value < 0.05 . TIC-enriching conditions led to down regulation of genes encoding extracellular matrix proteins, TREM1 signaling ($p < 10^{-4}$), and the NF- κ B complex ($p < 10^{-8}$; Figure 1A and 1B). Conversely these conditions led to an up regulation of genes encoding SMAD 1/5/8 and ID (inhibitor of DNA binding) family transcription factors ($p < 10^{-6}$; Figure 1A and 1C). We confirmed that TIC culture conditions led to differential regulation of NF- κ B genes in two ovarian cancer cell lines (Figure 1D). Notably, CXCL1 and CXCL2 were down regulated in both cell lines grown in TIC culture conditions. We have previously shown that these cytokines are regulated by classical NF- κ B signaling in ovarian cancer (6). CD44, PAX8, GAD1 and TFP12 have known NF- κ B binding sites in their promoter regions (21–23). We show here that they are also differentially expressed in TIC-enriching conditions, suggesting a potential role for NF- κ B in regulating the transition between tumor-initiating and proliferative cell behavior.

Next we examined changes in mRNA and protein levels of both classical and alternative NF- κ B transcription factors, in a panel of ovarian cancer cell lines grown in TIC-enriching conditions compared with traditional adherent conditions. Two of six cell lines tested showed a significant increase in the transcript level of *RELA*, the gene encoding the classical NF- κ B transcription factor p65 and four of six lines showed a significant increase in the transcript level of *RELB*, the gene encoding the alternative NF- κ B transcription factor RelB (Figure 2A). Moreover, relative to A culture, T culture led to 1.5 fold increase in

protein levels of RelB in seven out of nine ovarian cancer lines, including three primary lines (Figure 2B).

We used confocal microscopy to evaluate differences in cellular intensity of both RelA and RelB in primary ovarian cancer cells (VTCT) obtained from patient ascites. These experiments show both RelA and RelB mean cellular intensities are significantly increased in the spheroids grown in TIC conditions (Figure 2C) relative to cells grown in adherent conditions in agreement with the western blot findings. The same trend was observed for nuclear intensities. The enhanced fluorescence intensities measured for RelB far exceeded that measured for RelA. Our expression profiling data suggest RelA target genes are less active in the TIC conditions relative to the adherent conditions. Using an imaging flow cytometer we evaluated the localization of RelA as nuclear or cytoplasmic in ovarian cancer cells expressing the ALDH protein in adherent and TIC growth conditions. Co-staining with DAPI, we measured the localization of RelA in ALDH-positive cells. RelA distribution was both nuclear and cytoplasmic in either growth condition, but there was a decrease in the percentage of cells with nuclear RelA in TIC conditions compared with adherent conditions (Figure 2D). This suggests that RelA is more active in the nucleus of cells growing in adherent conditions.

These data suggest that alternative NF- κ B signaling may be more important for supporting cells with TIC features. To further explore this possibility and clarify the roles of classical versus alternative NF- κ B signaling we stably transfected ACI23, OV90 and VTCT ovarian cancer cells with inducible shRNAs targeting RELA or RELB and assessed the functional significance of these proteins relative to non-targeting control shRNA (shNeg). We chose ACI23 and OV90 cell lines because in culture they closely resemble the morphology of primary cells obtained from patients; furthermore, they have an appreciable number of cells positive for both CD133 expression and ALDH activity, established markers for ovarian TICs (20,24). Greater than 70% diminution of RELA or RELB transcript levels, following 72 hours exposure to 1 μ g/ml doxycycline, was confirmed using qRT-PCR for each cell line (Figure 3A). Protein level decrease was confirmed by western blot. Efficiency of protein knockdown was comparable for both A and T cultures (Figure 3B). Loss of RELB had no effect on the levels of RelA, however loss of RELA led to a small decrease in the levels of the alternative signaling proteins, p52 and RelB. These are known target genes for classical NF- κ B.

Our prior studies implicate NF- κ B in ovarian cancer aggressiveness and show that RelA and RelB are co-expressed in ovarian cancer tumor specimens (6,7). We measured the role of RELA or RELB in tumor development in athymic nude mice by injecting ACI23 or OV90 cells stably transfected with inducible shRNAs (cultured in T conditions) by intraperitoneal (IP), subcutaneous or intrabursal routes. All mice were fed doxycycline chow for two days prior to inoculation and throughout the remainder of the study to ensure persistent knockdown of RELA or RELB. The shRNA constructs co-express RFP also under the inducible promoter, so that induction can be identified by immune-staining for this protein. When transduced cells were injected IP, dramatically fewer RFP-positive cells were present in mice inoculated with cells containing shRelB construct compared to mice injected with cells containing shRelA or shNeg constructs (Figure 3C). The IP model suggests that RelB

is required for tumor growth in the “un-attached” or suspension setting. We next used the ACI23 cells to monitor subcutaneous tumor formation as this model was previously shown to give enhanced tumorigenic activity in T cultures compared to A cultures (20). Tumor growth was significantly diminished with knockdown of RelB, although the effect was less than in the IP model (Figure 3D). To verify shRNA activity was maintained we resected tumors and assessed protein levels via western blot (Figure 3E).

We next used OV90 cells to monitor intrabursal tumor growth as we recently found these cells to be highly tumorigenic in this model (24). This model most closely mimics human ovarian cancer, where tumors begin in the ovary or fallopian tube and then metastasize throughout the abdomen. Compared to mice that received cells containing the negative control shRNA (shNeg), mice implanted with cells containing either the RELA or RELB shRNAs gained significantly less tumor burden as measured using tumor weights (Figure 3F). Invasive xenograft cells were only present in the contralateral PBS-injected control ovaries of mice inoculated with shNeg cells, and not in mice that received cells depleted of either RELA or RELB (Figure 3G). Moreover, the ability to metastasize to abdominal organs was significantly reduced by knockdown of either RELA or RELB (Figure 3H).

Tumor cell viability was significantly decreased *in vitro* for both ACI23 and OV90 cells with RELA knockdown but not with RELB knockdown (Figure 4A). These data are intriguing given the significant decrease in tumorigenesis observed *in vivo* with loss of either transcription factor in both cell lines. We investigated whether either of these transcription factors supports features associated with TICs. Spheroid formation efficiency was significantly decreased with loss of either RELA or RELB in both ovarian cancer cell lines (Figure 4B). Our previous experiment showed, however, that there was no change in viability or proliferation with RELB knockdown. Therefore, these data suggest that the absence of RELB prevents efficient formation of spheroids, even though the same number of viable cells is present.

We hypothesized that classical and alternative NF- κ B signaling might be relevant in different subpopulations of ovarian cancer cells in TIC-enriched cultures. It has been established that classical NF- κ B signaling supports an anti-apoptotic, proliferative phenotype that sustains tumorigenesis (6,25–27), however the mechanism of alternative NF- κ B signaling in supporting tumor growth is unclear. We tested whether RELA might support a more proliferative subpopulation as would be expected given the traditional role of RELA in tumorigenesis. Cells positive for the proliferation marker Ki67 were quantified by flow cytometry in each of the knockdown conditions. OV90 cells showed a significant loss of ki67 positive cells when RelA was depleted, but not with depletion of RelB (Figure 4C, right). A similar trend was observed in ACI23 cells that approached statistical significance (Figure 4C, left). We measured the mean fluorescence intensity of Ki67 and found that both cell lines had a significant decrease in intensity specifically with depletion of RelA relative to negative control shRNA (Figure 4D).

Chemotherapy resistance is another feature commonly associated with TICs (11,28–30). We first verified that TIC culture conditions enriched for cells with carboplatin resistance by measuring viability across a range of concentrations in ACI23 and OV90 cells (Figure 5A).

IC50 was calculated using CompuSyn software (Paramus, NJ). Culture in TIC conditions caused greater than two-fold increase in carboplatin IC50 for both cell lines.

CD133 expression and ALDH activity have been the most consistent markers of ovarian cancer stem cells (31–35). For this reason, we proceeded to measure changes in these markers upon exposure to carboplatin. Carboplatin treatment led to a significant increase in the percentage of cells containing both ALDH activity and CD133 expression in ACI23 cells but had little effect on the CD133-positive cells relative to vehicle treatment (Figure 5B). Carboplatin treatment of OV90 cells led to a significant increase in the percentage of cells with ALDH activity and no effect on CD133-positive cells (Figure 5B). Quantitation of ALDH fluorescence intensity was significantly increased with carboplatin treatment in both cell lines (Figure 5C). Taken together, these data suggest that ALDH activity in our system more reliably associates with chemotherapy resistance, and both are increased in conditions that enrich for tumorigenesis.

We analyzed the influence of RELA and RELB in supporting carboplatin resistance. Cells remaining after 6-hour exposure to 100 uM carboplatin were collected and the lysates analyzed for protein changes. RelB increased in both cell lines after this treatment, whereas no obvious change was evident in total or phosphorylated RelA (Figure 5D). Viability assays demonstrate that loss of RELB was relatively better at restoring sensitivity to carboplatin (Figure 5E), again highlighting a key function of RelB in promoting the chemoresistant phenotype of TICs.

Flow cytometry experiments confirmed that loss of RELB significantly reduced the ALDH +CD133+ population *in vitro* for both ACI23 and OV90 cells (Figure 6A and 6B). Interestingly, depletion of either RELA or RELB led to a statistically significant diminution of TICs in cells derived from *in vivo* tumors (Figure 6C). It is important to note that the duration of knockdown in the *in vivo* tumors was substantially longer than the *in vitro* experiments (6 weeks versus 6 days). Given the known dynamic interplay between classical and alternative NF- κ B signaling, it is difficult to pinpoint an initial effect in the long-term setting.

Since ALDH is associated with chemotherapy resistance in our model and we showed RelB sensitizes ovarian cancer cells to chemotherapy, we analyzed ALDH fluorescence intensity after knockdown. Loss of RelB, but not RelA, led to a significant decrease in ALDH fluorescence intensity in cells obtained from both *in vitro* and *in vivo* conditions (Figure 6D). The VTCF primary ovarian cancer cells have decreased ALDH intensity with loss of RelB that approaches significance relative to the negative control shRNA. Even with the limited expansion imposed by primary cells in culture, and short term selection and induction periods, the decrease in ALDH activity with RELB loss was notable, indicating an important biological effect of this pathway.

Taken together our data suggest that both RELA and RELB support cells with TIC phenotypes, and point towards a greater influence of RELB on spheroid formation, chemoresistance and ALDH activity. We questioned whether the transcription factor RelB regulated expression of genes linked to stem cell functions. We first quantitated gene

expression levels of two ALDH isozymes and three stem cell transcription factors in cells grown in adherent or TIC-enriching conditions. There was significantly enriched expression of *ALDH1A2*, *OCT4*, and *NANOG* in OV90 cells grown in TIC conditions (Figure 7A). Gene expression in cells sorted for ALDH activity (ALDH+) show a significant enrichment in RelB, ALDH1A1, and ALDH1A2 expression in both cell lines relative to cells lacking ALDH activity (ALDH-) (Figure 7B). Differences in gene expression after knockdown of RELA or RELB further implicate RELB in regulating *ALDH1A2* gene expression (Figure 7C). Loss of RELA significantly decreased *NANOG* expression, however loss of RELB significantly decreased both *NANOG* and *ALDH1A2*, and neither affected *ALDH1A1*. Furthermore RELB knockdown significantly decreased expression of putative TIC gene CD44, consistent with previously published data showing that this gene is regulated by RelB in other cell types (22). Western blot confirmed RelB knockdown led to a decrease in ALDH1A2 protein levels (Figure 7D).

We next inquired whether alternative NF- κ B signaling through the RelB transcription factor directly binds to DNA in the ALDH1A2 promoter region. Chromatin immunoprecipitation was performed to compare RelA and RelB binding enrichment at the ALDH1A1 or ALDH1A2 promoter in TIC cultures relative to adherent cultures. Indeed, almost 3-fold enrichment of RelB binding occurred in a region approximately 2000 base pairs upstream of the transcription start site of ALDH1A2 that contains NF- κ B consensus sequence (Figure 7E). As expected based on qPCR results, enrichment of RelB at the promoter of ALDH1A1 was not significantly different from adherent cultures (Figure 7E). No enrichment of RelA was observed at the promoter of either ALDH1A1 or ALDH1A2 (Supplemental Figure 1). These data support a divergent role for alternative NF- κ B signaling in the regulation of ALDH1A2 in ovarian cancer TICs.

We confirmed the role of ALDH1A2 in supporting TIC features in ovarian cancer cells. Treatment with siRNA directed against ALDH1A1 or ALDH1A2 resulted in a significant decrease in the percentage of cells with ALDH activity relative to cells treated with non-targeting control siRNA (Figure 7F). Sensitization to carboplatin was significantly enhanced with knockdown of ALDH1A1 and nearly significantly with knockdown of ALDH1A2 (Figure 7G). Spheroid formation efficiency was significantly decreased with loss of ALDH1A2, whereas spheroid area was significantly decreased with knockdown of either ALDH1A1 or ALDH1A2 (Figure 7H).

DISCUSSION

Ovarian cancer is unique in that it spreads early through the shedding of malignant cells into the abdominal cavity, rather than invasion into blood vessels or lymphatics. In many cases, initial treatment of the disease appears successful, but then cancer recurs months to years after an apparent complete remission. This clinical pattern suggests that chemotherapy resistant cells are present at the onset, and capable of regenerating tumors. TICs are defined as cells that resist chemotherapy and can repopulate tumors after a period of apparent remission. Many investigators have attempted to replicate and study these putative TICs in the laboratory. The early shedding of ovarian cancer suggests that culturing cells in low attachment conditions may more accurately mimic the *in vivo* conditions for ovarian cancer

growth, dispersion and heterogeneity. We previously showed that such conditions resulted in formation of spheroid structures *in vitro*, and enhanced the ability of ovarian cancer cells to form tumors in mice. In this study, we employed low attachment growth conditions to enrich TIC spheroids and examined the functional consequences of classical and alternative NF- κ B transcription factor signaling in supporting TIC features.

We discovered that both classical and alternative NF- κ B pathways are important in sustaining TIC spheroids, with distinct roles. Classical NF- κ B signaling appears to promote proliferation and expansion of the spheroid, which is likely to be crucial for repopulation of the tumor recurrence. Classical NF- κ B signaling has been shown to support ovarian TICs (identified by expression of cell surface markers CD133 and CD44) through mechanisms including chemoresistance, epithelial-to-mesenchymal transition, invasion and migration potential (36–39). A recent study also linked classical NF- κ B activity to lipid metabolism in cancer spheroids (40). Our data complement and expand on this paradigm, by indicating that the alternative NF- κ B pathway supports self-renewal properties of the spheroid and directly or indirectly regulates genes linked to stem cell features such as ALDH1A2. In this study, we sought to understand the role of NF- κ B in regulating TICs defined by expression of CD133 and ALDH activity, a combination we and others have used to identify tumorigenic ovarian cancer subpopulations (20,31,32,41). This study further defines NF- κ B signaling in ovarian cancer by highlighting a novel role for alternative NF- κ B signaling in supporting tumorigenesis via regulation of ALDH⁺ chemoresistant subpopulations. Our data also underscore a role for the ALDH1A2 enzyme in supporting a TIC phenotype comparable to the ALDH1A1 enzyme already established in ovarian cancer (40–42). These findings expand on recent studies implicating ALDH1A2 in drug resistance and pathogenesis of ovarian cancer and reveals the diversity of ALDH enzymes in supporting cancer cells (43–46). We show that RelB regulates ALDH1A2 expression that is enhanced in culture conditions that enrich TICs. Moreover our work suggests that RELA more strongly supports proliferative subpopulations compared to RELB. Most likely, NF- κ B signaling is nuanced in different subpopulations of cells. This idea is intriguing given the increasing evidence of intratumor heterogeneity and the dynamic relationships between cellular subclones that cooperate to develop malignancies, resist therapy and repopulate tumors (3).

TICs must be eliminated with initial treatment in order to prevent relapse of ovarian cancer. NF- κ B may be a key target in TICs. Our study suggests that both classical and alternative NF- κ B signaling is present in the heterogeneous spheroid (Figure 7I). Specific inhibition of downstream effectors of NF- κ B in spheroids may eliminate TICs. ALDH enzyme activity is a common marker of cancer stem cells in a variety of tissues and is associated with high tumor-initiating capacity of ovarian cancer cells and poor outcomes for patients with ovarian cancer (32,47–49). Selective inhibitors of ALDH are in development and may provide a therapeutic benefit for tumor cells dependent on ALDH activity (42,48,50), particularly if used in combination with traditional chemotherapy. This study highlights how differential NF- κ B signaling can maintain diverse phenotypes within the cancer spheroid. Further exploration into the dynamic biology of NF- κ B in ovarian cancer spheroids will increase our understanding of the complex nature of these tumor-initiating structures, with the hopes of identifying additional biology to influence a long-term cure for women with this disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Differential NF- κ B signaling in TIC-enriching culture conditions

A) Up- and downregulated genes were examined in ACI23 cells cultured in TIC and adherent conditions. Heat map summarizes genes with fold change >2 and p-value <0.05 in either direction (red = up; green = down) from three biological replicates. **B)** IPA analysis designated the NF- κ B complex as a top upstream regulator. NF- κ B predicted to be inhibited in TIC cultures relative to adherent cultures. **C)** The third ranked enriched canonical pathway identified using IPA, embryonic stem cell pluripotency, indicates increased SMAD and ID signaling in TIC cultures relative to adherent cultures. **D)** qRT-PCR of selected NF- κ B regulated genes relative to GAPDH housekeeping gene in ACI23 and OV90 ovarian

cancer cell lines grown in TIC-enriching conditions for 5 days relative to cells grown in traditional adherent cultures. Data shown represent average and SEM from 3 independent experiments * $p < 0.05$, Student's t test.

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Figure 2. Baseline expression of classical and alternative NF- κ B transcription factors
A) Gene expression fold changes of RELA and RELB in a panel of ovarian cancer cell lines grown in TIC-enriching conditions for 5 days relative to cells grown in traditional adherent cultures. Data shown represent average and SEM from 3 independent experiments * $p < 0.05$, Student's t test. B) Western blot showing baseline protein levels of classical NF- κ B transcription factor RelA (p65) and alternative NF- κ B transcription factors, p100/52 and RelB in adherent (A), TIC (T) or floater (F) cultures. Densitometric analysis of protein level fold changes in F or T culture relative to A culture is shown in the heat map. C) Confocal

microscopy examining total cellular and nuclear staining of RelA and RelB in adherent (A) or TIC (T) conditions. Representative images shown at bottom. Box plots represent quantitation of at least 500 cells for each condition. Error bars indicate standard deviations. p-values for cellular RelA $< 3.4 \times 10^{-5}$ and cellular RelB $< 2.0 \times 10^{-218}$ and nuclear RelA $< 6.6 \times 10^{-32}$ and nuclear RelB $< 1.2 \times 10^{-219}$ as determined by paired *t*-tests. Scale bar = 10 μm . **D)** Imaging flow cytometer evaluation of RelA in TIC (T) or adherent (A) conditions. Representative images of nuclear and cytoplasmic RelA shown on top. Histograms demonstrate change in localization analyzed using similarity feature. Bar graph represents quantitation of 10,000 cells per sample.

Figure 3. RelA and RelB support ovarian cancer tumor formation

A) Gene expression fold changes of RELA and RELB after stable transfection of ACI23 and OV90 ovarian cancer cell lines with inducible shRNAs against RelA (shRelA), RelB (shRelB) or non-targeting control transcript (shNeg). Data shown represents average fold changes and SEM relative to non-targeting negative control shRNA from 3 independent experiments * p<0.05, Student's t test. **B)** Whole-cell lysate western blot showing protein level decrease after 72 hours' exposure to 1 ug/ml doxycycline in adherent (A) and TIC-enriched (T) cultures. **C)** Intraperitoneal injection of 1×10^6 OV90 cells stably transfected

with shRNAs. Immunohistochemical analysis of RFP positive cells in resected organs showing macrometastases. **D)** Subcutaneous injection of 1×10^6 ACI-23 cells stably transfected with shRNAs. Tumors were monitored and measured twice weekly. Mice were sacrificed 24 days following tumor cell inoculation. Shown is the average tumor volume and SEM for each group of 5 mice from 3 independent experiments. Representative images of resected tumors are shown. Scale bar = 1cm. At day 24, $*p=0.0047$ for RelA and 0.0001 for RelB, two-way ANOVA with Dunnett post-hoc test. Differences were not significant at any other time points. **E)** Single-cell suspensions of resected tumors were analyzed by western blot for RelA and RelB to confirm knockdown of protein was maintained. **F)** Intrabursal injection of 2×10^4 OV90 cells stably transfected with shRNAs into the intrabursal sac of 10 mice. Mice were weighed twice weekly and sacrificed 60 days after tumor cell inoculation. Ovaries were resected and weighed. Shown are the average tumor weights and SEM for each group of 5 mice from 2 independent experiments. Representative images of xenograft ovaries are shown. Scale bar = 1 cm. $*p=0.0181$ for RelA and 0.0187 for RelB, one-way ANOVA with Dunnett post-hoc test. **G)** H&E stained ovary sections were analyzed by a pathologist for evaluation of invasiveness. Representative images of invasive and non-invasive xenograft observed in shNeg samples and no xenograft observed in shRelA sample. Scale bar = 300 μ m **H)** In addition to ovaries, sections of liver, pancreas, peritoneal wall, and diaphragm were removed at necropsy and fixed in 4% paraformaldehyde. Tissues were stained with H&E and analyzed by a pathologist for metastatic spread in all ten mice for each condition. Metastases were present in contralateral ovaries, diaphragm, pancreas, peritoneal wall, and liver in the shNeg mice. No metastases were seen with shRelA mice. Only contralateral metastases were seen in one shRelB mouse $*p<0.05$, Chi-square test.

Figure 4. RelA and RelB differentially support viability but equally support spheroid formation

A) ACI23 and OV90 cells were grown in TIC conditions for 72 hours. 2×10^3 viable cells were seeded in ultra-low attachment 96-well plates and allowed to grow under doxycycline induction for 72 hours. Viability was calculated relative to the negative control shRNA.

* $p=0.0017$ for RelA in ACI23 and 0.0001 for RelA in OV90, one-way ANOVA with Dunnett post-hoc test. **B)** 5×10^2 cells were seeded in ultra-low attachment 96-well plates with TIC culture media and cultured for 7 days. Three image fields were obtained for each well. * $p=0.0116$ for RelA and 0.0005 for RelB in ACI23; $p=0.0007$ and 0.0251 for RelB in

OV90, one-way ANOVA with Dunnett post-hoc test. **C)** ACI23 and OV90 cells grown in TIC conditions for 72 hours before exposure to doxycycline for an additional 72 hours. Cells were fixed, permeabilized, and stained with Ki67 antibody conjugated to Alexa 647 fluorophore and quantitated by flow cytometry. * $p=0.0178$ for RelA in OV90, one-way ANOVA with Dunnett post-hoc test. **D)** Fold change in geometric mean Ki67 fluorescence intensity in shRNA expressing cells. Representative histograms shown on left. * $p=0.191$ for ACI23 and 0.0149 for OV90 cells, Kruskal-Wallis test. Data shown for all panels represent average and SEM from 5 independent experiments.

Figure 5. RelB supports resistance to carboplatin

A) 2×10^3 viable cells were seeded in adherent (A) or ultra-low attachment (T) 96-well plates with traditional or stem cell media, respectively. The next day a range of carboplatin concentrations was added and cells were cultured for 72 hours. Viability was calculated relative to vehicle control. **B)** 1×10^6 AC123 and OV90 cells were seeded in adherent plates and allowed to attach overnight. The next day 30 μM of carboplatin was added and cells were cultured an additional 48 hours. CD133 expression and ALDH activity was quantitated by flow cytometry and assessed relative to vehicle control. * $p < 0.0001$ for ALDH⁺CD133⁺

in ACI23; $p < 0.0231$ for ALDH+ in OV90, one-way ANOVA with Dunnett post-hoc test **C)** Fold change in geometric mean of ALDH fluorescence with carboplatin treatment significantly increased relative to vehicle control. * $p = 0.008$ for ACI23; $P = 0.002$ for OV90, Mann-Whitney test. **D)** Top, Cells were seeded in adherent conditions and allowed to attach overnight. Cells were then treated with 100 μM of carboplatin for 6 hours followed by whole cell lysate preparation and western blot. Bottom, densitometric analysis relative to vehicle for three independent blots. **E)** 2×10^3 viable cells grown TIC conditions for 72 hours were seeded ultra-low attachment 96-well plates with doxycycline. The next day 30 μM of carboplatin was added and cells were cultured for 72 hours. Viability was calculated relative to vehicle control for each shRNA group. * $p < 0.03$ for RelB in ACI23; * $p < 0.008$ for RelB in OV90; # $p < 0.016$ for RelA in OV90, one-way ANOVA with Dunnett post-hoc test. Bar graphs for all panels represent average and SEM obtained from at least three independent experiments.

Figure 6. RelB supports CD133+ALDH+ ovarian cancer cells

A) ACI23 cells were grown in TIC conditions for 72 hours before addition of doxycycline for an additional 72 hours. Cells were analyzed using flow cytometry by first gating on the RFP channel to select the shRNA expressing cells and subsequent quantitation of CD133 expression and ALDH activity. Data shown are average and SEM percent double-positive cells relative to negative control shRNA from 5 independent experiments. * $p=0.0244$, one-way ANOVA with Dunnett post-hoc. **B)** OV90 cells were treated and analyzed as in (A). * $p=0.0070$, one-way ANOVA with Dunnett post-hoc. **C)** Single-cell suspensions of ACI23

xenograft tumors were freshly prepared and analyzed by flow cytometry as in (A). Data shown represent average and SEM obtained from tumors resected from 3 mice in each group. * $p=0.0382$ for RelA and 0.0152 for RelB, one-way ANOVA with Dunnett post-hoc. **D)** Fold change in geometric mean of ALDH fluorescence in shRNA expressing cells. ALDH activity significantly decreased with loss of RelB relative to control shRNA. Representative histograms shown for ACI23 and OV90 cells. Bar graphs represent average and SEM obtained from at least three independent experiments. * $P=0.007$ for ACI23; $P=0.029$ for OV90; $P=0.019$ for ACI23 xenograft derived cells; # $P=0.140$ for VTCTF, Kruskal-Wallis test.

Figure 7. RelB regulates expression of ALDH1A2

A) qRT-PCR of selected stem cell genes in ACI23 and OV90 ovarian cancer cell lines cultured in TIC-enriching conditions for 5 days relative to cells cultured in adherent conditions. * p<0.05, Student's t test. **B)** qRT-PCR for RELA, RELB, and ALDH genes in ALDH positive cells. Data shown is average fold enrichment and SEM relative to ALDH negative cells for three independent experiments. * p<0.05, Student's t test. **C)** OV90 cells were grown in TIC conditions for 72 hours before addition of doxycycline for an additional 72 hours. RNA was collected and gene expression analyzed by qRT-PCR. Data shown is

average fold enrichment and SEM relative to negative shRNA. * $p < 0.05$ one-way ANOVA with Dunnett's post-hoc. **D**) Whole-cell lysate western blot analysis showing protein level decrease after 72 hours' exposure to doxycycline. The western blot shown from Figure 2 was re-probed using ALDH antibodies. **E**) Left, TIC conditions significantly enhanced binding of RelB to specific NF- κ B consensus sites in the ALDH1A2 promoter as assessed by ChIP-PCR. X-axis represents distance in base pairs from transcription start site. There is no significant binding enrichment under adherent conditions. Enrichment is defined as percent input. Data shown is average fold enrichment relative to the negative control IgG antibody pull down. Right, TIC conditions did not significantly enrich binding of RelB to any NF- κ B consensus sites in the ALDH1A1 promoter. * $p < 0.002$, Student's t test. **F**) OV90 cells transfected with siRNAs targeting ALDH1A1, ALDH1A2 or a non-targeting negative control sequence were analyzed by flow cytometry for ALDH activity. * $p < 0.05$ one-way ANOVA with Dunnett's post-hoc **G**) 2×10^3 OV90 cells transfected with indicated siRNAs were seeded in 96-well plates with 30 μ M of carboplatin. After 72 cell death was calculated relative to vehicle control for each siRNA group. * $p < 0.05$ one-way ANOVA with Dunnett's post-hoc **H**) 5×10^2 cells transfected with indicated siRNAs were seeded in ultra-low attachment 96-well plates with TIC culture media and cultured for 5 days. Three image fields were obtained for each well. * $p < 0.05$ one-way ANOVA with Dunnett's post-hoc test. **I**) Schematic illustrating the NF- κ B signaling mechanisms supporting ovarian cancer spheroids. Data shown for all panels represent average and SEM for 3 independent experiments.