NFκB Promotes Ovarian Tumorigenesis via Classical Pathways That Support Proliferative Cancer Cells and Alternative Pathways That Support ALDH+ Cancer Stem–like Cells

Carrie D. House1, Elizabeth Jordan1, Lidia Hernandez2, Michelle Ozaki1, Jana M. James1, Marianne Kim1, Michael J. Kruhlak2, Eric Batchelor3, Fathi Elloumi4, Margaret C. Cam4, and Christina M. Annunziata1

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 NFκB 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 NFκB 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 NFκB sustains diverse cancer phenotypes via distinct classical and alternative signaling pathways, with implications for improved understanding of disease recurrence and therapeutic response. Cancer Res; 77(24); 6927–40. ©2017 AACR.

Introduction

Ovarian cancer is the most lethal gynecologic malignancy in the United States, 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). Disease recurrence is a major cause of morbidity and mortality, and mechanisms of tumor relapse and chemoresistance are unclear. Evidence suggests tumor-initiating cells (TIC) 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 NFκB signaling are required for aggressive tumor behavior and metastatic potential in subsets of ovarian cancers (6–8). NFκB signaling is a complex network that follows both classical (canonical) and alternative (noncanonical) cascades, defined by upstream kinases and ultimate activation of either p65/p50 (classical) or RelB/p52 (alternative) transcription factors. Elevated classical NFκB signaling has been observed in TICs of prostate, breast, and ovarian tumors (9–11); however, the role of alternative NFκB signaling is not established. Both cascades are required for maintenance and promotion of breast cancer TICs (12). Classical and alternative NFκB pathways can regulate each other as well as integrate with other signaling pathways for fine-tuning functional outputs (13–16). NFκB 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 NFκB suggest that 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 NFκB 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 NFκB supports proliferative cells, whereas alternative NFκB supports cells with low proliferative potential–bearing TIC markers. This study reveals a novel role for RelB in ovarian cancer and demonstrates mechanistically that NFκB 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 Supplementary Table S1. OVCAR8 (HTB-161), CAOV3 (HTB-75), and OV90 (CRL-11732) were obtained directly from the ATCC and maintained in RPMI medium supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine and penicillin–streptomycin (Gibco) 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 Modified Eagle Nutrient Mixture F-12.
Baseline expression of classical and alternative NFκB 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 three independent experiments; *P < 0.05, Student t test. B, Western blot analysis showing each condition. Error bars, SDs. Densitometric analysis of protein level quantitation of at least 500 cells for shown at bottom. Box plots represent quantitation of 10,000 cells per sample.

(DMEM/F12) medium (Gibco) supplemented with 10% FBS, 2 mmol/L 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. Deidentified 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 [82x341]fl asks (Corning) with stem cell media: DMEM: F12 (l-glutamine, +15 mmol/L HEPES; Gibco) supplemented with 1% penicillin/streptomycin, 1% KnockOut serum replacement (Gibco), 0.4% BSA (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 to 3 days for a final concentration of 20 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 dosilycine chow (200 mg/kg) for the duration of the study. OV90 and ACI23 cells were maintained in TIC-enriching conditions for one week before injections. For intraperitoneal xenografts, 1 x 10⁶ OV90 cells in a volume of 500 μL PBS were injected into the abdominal cavity of 8-week-old female athymic Nu/Nu mice. Tumor burden for mice injected intraperitoneally was assessed by pathology after necropsy and collection of organs. For subcutaneous xenografts, 1 x 10⁶ ACI23 cells in 500 μL were subcutaneously injected into the right flank of 8-week-old female athymic Nu/Nu mice. For controls, 500 μL 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 x 10^6 OV90 cells in 5 µL were injected into the right bursa of 8-week-old female athymic Nu/Nu mice. For controls, 5 µL 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 and analyzed by a pathologist.

RNA extraction and quantitative real-time PCR
Total RNA was isolated and purified using the RNaseasy Mini Kit (QIAGEN) as per the 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 quantitative real-time PCR (qRT-PCR). Analysis of gene expression was performed on the ABI7 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 ΔΔCt. Catalog numbers for commercial primers are provided in Supplementary Table S2.

Gene expression
Total RNA was isolated from three independent cultures of ovarian cancer cells grown on traditional or ultralow 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 (1 µg) 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). Datasets are available on the GEO repository (GSE94358).

Western blot analysis
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 used to determine protein concentrations. 30 µg 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 Technology (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 spheres 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 mono-valent Fab fragment goat anti-rabbit secondary (Jackson Immunoresearch; 1:50 1 hour at room temperature), Alexafluor 647 Ki67 rabbit antibody (BioLegend; 1:50 overnight at 4 degrees), Alexafluor 555 RelB rabbit antibody (Bioss; 1:100 1 hour at room temperature), and Alexafluor 488 p65 rabbit antibody (Abcam; 1:100 1 hour at room temperature). Cells were washed with PBS between each antibody staining. Finally, cells were stained with DAPI (Thermo Scientific; 1:1,000) 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 (NA. 1.4) oil immersion objective lens (Carl Zeiss Microscopy). Image acquisition details can be found in Supplementary Experimental Procedures. Further quantification and statistical analysis of fluorescence levels in populations of individual cells was performed using custom-written software in Matlab (Mathworks).

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 nontargeting control transcript (shNeg). Data shown represent average fold changes and SEM relative to nontargeting negative control shRNA from three independent experiments. *, P < 0.05, Student’s t test. B, Whole-cell lysate Western blot analysis showing protein level decrease after 72-hour exposure to 1-fold changes and SEM relative to nontargeting negative control shRNA from three independent experiments. Differences were not significant at any other time points. C, Single-cell suspensions of resected tumors were analyzed by Western blot analysis for RelA and RelB to confirm knockdown of protein was maintained. D, Intraperitoneal injection of 2 x 10^6 OV90 cells stably transfected with shRNAs into the intraperitoneal sac of 10 mice. Mice were sacrificed 24 days following tumor cell inoculation.Shown is the average tumor volume and SEM for each group of 5 mice from three independent experiments. Representative images of resected tumors are shown; scale bar, 1 cm. 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 analysis for RelA and RelB to confirm knockdown of protein was maintained. F, Intraperitoneal injection of 2 x 10^6 OV90 cells stably transfected with shRNAs into the intraperitoneal sac of 10 mice. Mice were sacrificed 65 days following 24 days tumor cell inoculation. Ovaries were resected and weighed. Shown are the average tumor weights and SEM for each group of 5 mice from two 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, Hematoxylin and eosin–stained ovary sections were analyzed by a pathologist for evaluation of invasiveness. Representative images of invasive and noninvasive xenograft observed in shNeg samples and no xenograft observed in shRelA sample. Scale bar, 500 µm. 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 hematoxylin and eosin and analyzed by a pathologist for metastatic spread in all 10 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, χ^2 test.
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 EF1α promoter and red fluorescent protein (TurboRFP) reporter gene. OV90, ACI23 and VTF603 cells were transduced according to the manufacturer’s protocol. Briefly, cells were transduced with viral particles in serum-free transduction media for 24 hours followed by selection with 2 μg/mL puromycin for 7 days. For transient gene knockdown studies On-Target Plus Smartpool siRNAs were purchased from Dharmacon for ALDH1A1 (catalog no. L-008722-00), ALDH1A2 (catalog no. L-008118-00) and non-targeting control (catalog no. D-001810-10-20). Briefly, cells were seeded at 60% confluence and transfected with 20 nmol/L siRNA for 24 hours before proceeding with functional assays.

Flow cytometry

ALDH activity was evaluated using the Aldefluor Kit (StemCell Technologies) according to the 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 μL 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 magnification using Axio Observer A1 Inverted Microscope and analyzed using ImageJ Software 1.48v. Spheroids with diameter ≥50 μm were quantified. Spheroid efficiency was calculated as spheroids ≥50 microns/cells per well.

Cell viability assay

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

Chromatin immunoprecipitation-qPCR assay

The SimpleChIP Enzymatic Chromatin IP Kit (magnetic beads) was purchased from Cell Signaling Technology and assays performed according to the manufacturer’s instructions. Antibodies for RelB were also from Cell Signaling Technology (catalog #10514). Promo was used to evaluate DNA sequences for transcription factor binding sites (http://alggen.lsi.usc.es/cgi-bin/promo/mv3/promo/prominin1.cgi?dirDB=TF_8.3). The first 5,000 bases upstream of the transcription start site were screened for binding motifs that correspond to NFκB consensus-binding sequence. The quantification of transcription factor binding to target genes was calculated by measuring the ratio of chromatin immunoprecipitation (ChIP)-to-Input and the normal rabbit IgG antibody served as a negative control. All primers used for ChIP-qPCRs are listed in Supplementary Table S2.

Statistical analysis

Raw microarray (CEL) data were 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 NFκB 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 on the basis of cutoff values of fold change >2.0 and P < 0.05. TIC-enriching conditions led to down-regulation of genes encoding extracellular matrix proteins, TREM1 signaling (P < 10^{-4}), and the NFκB complex (P < 10^{-6}; Fig. 1A and B). Conversely these conditions led to up-regulation of genes encoding SMAD 1/5/8 and ID (inhibitor of DNA binding) family transcription factors (P < 10^{-5}; Fig. 1A and C). We confirmed that TIC culture conditions led to differential regulation of NFκB genes in two ovarian cancer cell lines (Fig. 1D). Notably, CXCL1 and CXCL2 were downregulated in both cell lines grown in TIC culture conditions. We have previously shown that these cytokines are regulated by classical NFκB signaling in ovarian cancer (6). CCL4, CXCL8, GAD1, and TIP47 have known NFκB–binding sites in the promoter regions (21–23). We show here that they are also differentially expressed in TIC-enriching conditions, suggesting a potential role for NFκB 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 NFκB 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 NFκB transcription factor p65 and four of six lines showed a significant increase in the transcript level of RELB, the gene encoding the alternative NFκB transcription factor RelB (Fig. 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 (Fig. 2B).

We used confocal microscopy to evaluate differences in cellular intensity of both RelA and RelB in primary ovarian cancer cells (VTCF) obtained from patient ascites. These experiments show both RelA and RelB mean cellular intensities are significantly increased in the spheroids grown in TIC conditions (Fig. 2C) relative to cells grown in adherent conditions in agreement with
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. A total of $2 \times 10^5$ viable cells were seeded in ultralow 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, A total of $5 \times 10^2$ cells were seeded in ultralow 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 are 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 five independent experiments. ns, nonsignificant.
Figure 5.
RelB supports resistance to carboplatin. A, A total of $2 \times 10^3$ viable cells were seeded in adherent (A) or ultralow 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, A total of $1 \times 10^6$ ACI23 and OV90 cells were seeded in adherent plates and allowed to attach overnight. The next day, 30 $\mu$mol/L of carboplatin was added and cells were cultured for 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 $\mu$mol/L of carboplatin for 6 hours, followed by whole-cell lysate preparation and Western blot analysis. Bottom, densitometric analysis relative to vehicle for three independent blots. E, A total of $2 \times 10^3$ viable cells grown in TIC conditions for 72 hours were seeded in ultralow attachment 96-well plates with doxycycline. The next day, 30 $\mu$mol/L 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 five 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 three 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 VTCF, Kruskal–Wallis test. ns, nonsignificant.
Alternative NFκB Regulates ALDH<sup>+</sup> Ovarian Cancer Cells

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 that 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 ALDH1 protein in adherent and TIC growth conditions. Containing 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 (Fig. 2D). This suggests that RelA is more active in the nucleus of cells growing in adherent conditions.

These data suggest that alternative NFκB signaling may be more important for supporting cells with TIC features. To further explore this possibility and clarify the roles of classical versus alternative NFκB signaling, we stably transfocused AC123, OV90, and VTIC ovarian cancer cells with inducible shRNAs targeting RELA or RELB and assessed the functional significance of these proteins relative to nontargeting control shRNA (shNeg). We chose AC123 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 μg/mL doxycycline, was confirmed using qRT-PCR for each cell line (Fig. 3A). Protein level decrease was confirmed by Western blot analysis. Efficiency of protein knockdown was comparable for both A and T cultures (Fig. 3B). Loss of RELA 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 NFκB.

Our prior studies implicate NFκB in ovarian cancer aggressiveness and show that RelA and RelB are coexpressed in ovarian cancer tumor specimens (6, 7). We measured the role of RELA or RELB in tumor development in athymic nude mice by injecting AC123 or OV90 cells stably transfocused with inducible shRNAs (cultured in T conditions) by intraperitoneal, subcutaneous, or intrabursal routes. All mice were fed doxycycline chow for two days before inoculation and throughout the remainder of the study to ensure persistent knockdown of RELA or RELB. The shRNA constructs coexpress RFP also under the inducible promoter, so that induction can be identified by immune-staining for this protein. When transduced cells were injected intraperitoneally, dramatically fewer RFP-positive cells were present in mice inoculated with cells containing shRelB construct compared with mice injected with cells containing shRelA or shNeg constructs (Fig. 3C). The intraperitonal model suggests that RelB is required for tumor growth in the “unattached” or suspension setting. We next used the AC123 cells to monitor subcutaneous tumor formation as this model was previously shown to give enhanced tumorigenic activity in T cultures compared with A cultures (20).

Tumor growth was significantly diminished with knockdown of RelB, although the effect was less than in the intraperitoneal model (Fig. 3D). To verify shRNA activity was maintained we resected tumors and assessed protein levels via Western blot analysis (Fig. 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 with 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 (Fig. 3F). Invasive xenografts 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 (Fig. 3G). Moreover, the ability to metastasize to abdominal organs was significantly reduced by knockdown of either RELA or RELB (Fig. 3H).

Tumor cell viability was significantly decreased <i>in vitro</i> for both AC123 and OV90 cells with RELA knockdown but not with RELB knockdown (Fig. 4A). These data are intriguing given the significant decrease in tumorigenesis observed <i>in vivo</i> 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 (Fig. 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 NFκB signaling might be relevant in different subpopulations of ovarian cancer.
cells in TIC-enriched cultures. It has been established that classical NFκB signaling supports an antiapoptotic, proliferative phenotype that sustains tumorigenesis (6, 25–27); however, the mechanism of alternative NFκB 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 (Fig. 4C, right). A similar trend was observed in AC123 cells that approached statistical significance (Fig. 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 (Fig. 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 AC123 and Ov90 cells (Fig. 5A). IC_{50} was calculated using CompuSyn software. Culture in TIC conditions caused greater than 2-fold increase in carboplatin IC_{50} 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 AC123 cells but had little effect on the CD133-positive cells relative to vehicle treatment (Fig. 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 (Fig. 5B). Quantitation of ALDH fluorescence intensity was significantly increased with carboplatin treatment in both cell lines (Fig. 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 μmol/L 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 (Fig. 5D). Viability assays demonstrate that loss of RELB was relatively better at restoring sensitivity to carboplatin (Fig. 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 AC123 and Ov90 cells (Fig. 6A and B). Interestingly, depletion of either RELA or RELB led to a statistically significant diminution of TICs in cells derived from in vivo tumors (Fig. 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 vs. 6 days). Given the known dynamic interplay between classical and alternative NFκB signaling, it is difficult to pinpoint an initial effect in the long-term setting.

Because 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 (Fig. 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 toward 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 isoforms and three stem cell transcription factors in cells grown in adherent or TIC-enriching conditions. There was significantly enriched expression of ALDH1A1, OCT4, and NANOG in Ov90 cells grown in TIC conditions (Fig. 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^{-}; Fig. 7B). Differences in gene expression after knockdown of RELA or RELB further implicate RELB in regulating ALDH1A2 gene expression (Fig. 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 analysis confirmed that RelB knockdown led to a decrease in ALDH1A2 protein levels (Fig. 7D).

We next inquired whether alternative NFκB 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 2,000 base pairs upstream of the transcription start site of ALDH1A2 that contains NFκB consensus sequence (Fig. 7E). As expected on the basis of qPCR results, enrichment of RelB at the promoter of ALDH1A1 was not significantly different from adherent cultures (Fig. 7E). No enrichment of RelB was observed at the promoter of either ALDH1A1 or ALDH1A2 (Supplementary Fig. S1). These data support a divergent role for alternative NFκB 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 nontargeting control siRNA (Fig. 7F). Sensitization to carboplatin was significantly enhanced with knockdown of ALDH1A1 and nearly significantly with knockdown of ALDH1A2 (Fig. 7G). Spheroid formation efficiency was significantly decreased with loss of ALDH1A2, whereas spheroid area was significantly decreased with knockdown of either ALDH1A1 or ALDH1A2 (Fig. 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 chemoresistant 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 vitro 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 used low attachment growth conditions to enrich TIC spheroids and examined the functional consequences of classical and alternative NFκB transcription factor signaling in supporting TIC features.

We discovered that both classical and alternative NFκB pathways are important in sustaining TIC spheroids, with distinct roles. Classical NFκB signaling appears to promote proliferation and expansion of the spheroid, which is likely to be crucial for repopulation of the tumor recurrence. Classical NFκB 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 NFκB activity to lipid metabolism in cancer spheroids (40). Our data complement and expand on this paradigm by indicating that the alternative NFκB 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 NFκB 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 NFκB signaling in ovarian cancer by highlighting a novel role for alternative NFκB signaling in supporting tumorigenesis via regulation of ALDH1α chemoresistant subpopulations. Our data also underscore a role for the ALDH1A2 enzyme in supporting a TIC phenotype comparable with 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, NFκB 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 to prevent relapse of ovarian cancer. NFκB may be a key target in TICs. Our study suggests that both classical and alternative NFκB signaling is present in the heterogeneous spheroid (Fig. 7I). Specific inhibition of downstream effectors of NFκB in spheroids may eliminate TICs. ALDH1A2 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 NFκB signaling can maintain diverse phenotypes within the cancer spheroid. Further exploration into the dynamic biology of NFκB 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.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: C.D. House, C.M. Annunziata
Development of methodology: C.D. House, M. Kim
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C.D. House, L. Hernandez, M. Ozaki, J.M. James, M.J. Kruhlak
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C.D. House, E. Jordan, L. Hernandez, J.M. James, M. Kim, M.J. Kruhlak, E. Batchelor, F. Elloumi, M.C. Cam, C.M. Annunziata
Writing, review, and/or revision of the manuscript: C.D. House, E. Jordan, L. Hernandez, M. Ozaki, J.M. James

Supervision: C.M. Annunziata

Acknowledgments
We thank Karen Wolcott of the CCR FACS core facility, Elena Kuznetsova of the CCR mouse core facility, Brian Hall of Millipore Sigma, and Dr. Tobias Eggert for their excellent technical assistance.

Grant Support
This study was supported by the NCI intramural research program ZIA BC 011054 (to C.M. Annunziata) and 1K99CA204727 (to C.D. House).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received February 3, 2017; revised February 13, 2017; accepted October 20, 2017; published OnlineFirst October 26, 2017.

References

www.aacrjournals.org Cancer Res; 77(24) December 15, 2017 6939


NFκB Promotes Ovarian Tumorigenesis via Classical Pathways That Support Proliferative Cancer Cells and Alternative Pathways That Support ALDH+ Cancer Stem-like Cells


_Cancer Res_ 2017;77:6927-6940. Published OnlineFirst October 26, 2017.

**Updated version**
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-17-0366

**Supplementary Material**
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2017/10/25/0008-5472.CAN-17-0366.DC1

**Cited articles**
This article cites 49 articles, 12 of which you can access for free at:
http://cancerres.aacrjournals.org/content/77/24/6927.full#ref-list-1

**Citing articles**
This article has been cited by 2 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/77/24/6927.full#related-urls

**E-mail alerts**
Sign up to receive free email-alerts related to this article or journal.

**Reprints and Subscriptions**
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

**Permissions**
To request permission to re-use all or part of this article, use this link
http://cancerres.aacrjournals.org/content/77/24/6927.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.