IL-1-conferred gene expression pattern in ER$^+$ BCa and AR$^+$ PCa cells is intrinsic to ER$^-$ BCa and AR$^-$ PCa cells and promotes cell survival

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Abstract

Background: Breast (BCa) and prostate (PCa) cancers are hormone receptor (HR)-driven cancers. Thus, BCa and PCa patients are given therapies that reduce hormone levels or directly block HR activity; but most patients eventually develop treatment resistance. We have previously reported that interleukin-1 (IL-1) inflammatory cytokine downregulates ER$\alpha$ and AR mRNA in HR-positive (HR$^+$) BCa and PCa cell lines, yet the cells can remain viable. Additionally, we identified pro-survival proteins and processes upregulated by IL-1 in HR$^+$ BCa and PCa cells, that are basally high in HR$^-$ BCa and PCa cells. Therefore, we hypothesize that IL-1 confers a conserved gene expression pattern in HR$^+$ BCa and PCa cells that mimics conserved basal gene expression patterns in HR$^-$ BCa and PCa cells to promote HR-independent survival and tumorigenicity.

Methods: We performed RNA sequencing (RNA-seq) for HR$^+$ BCa and PCa cell lines exposed to IL-1 and for untreated HR$^-$ BCa and PCa cell lines. We confirmed expression patterns of select genes by RT-qPCR and used siRNA and/or drug inhibition to silence select genes in the BCa and PCa cell lines. Finally, we performed Ingenuity Pathway Analysis (IPA) and used the gene ontology web-based tool, GOrilla, to identify signaling pathways encoded by our RNA-seq data set.

Results: We identified 350 genes in common between BCa and PCa cells that are induced or repressed by IL-1 in HR$^+$ cells that are, respectively, basally high or low in HR$^-$ cells. Among these genes, we identified Sequestome-1 (SQSTM1/p62) and SRY (Sex-Determining Region Y)-Box 9 (SOX9) to be essential for survival of HR$^-$ BCa and PCa cells. Analysis of publicly available data indicates that p62 and SOX9 expression are elevated in HR-independent BCa and PCa sublines generated in vitro, suggesting that p62 and SOX9 have a role in acquired hormone receptor independence and treatment resistance. We also assessed HR$^-$ cell line viability in response to the p62-targeting drug, verteporfin, and found that verteporfin is cytotoxic for HR$^-$ cell lines.

Conclusions: Our 350 gene set can be used to identify novel therapeutic targets and/or biomarkers conserved among acquired (e.g. due to inflammation) or intrinsic HR-independent BCa and PCa.

Keywords: Interleukin-1, Breast cancer, Prostate cancer, p62/SQSTM1, Estrogen receptor, Androgen receptor
Background

Breast (BCa) and prostate (PCa) cancer share similar etiology, where hormone receptors (HR) drive cancer cell survival [1, 2]. Estrogen Receptor Alpha (ERα) promotes BCa tumor growth and Androgen Receptor (AR) promotes PCA tumor growth; thus, patients are treated with HR-targeting therapies. Unfortunately, patients can become treatment resistant due to loss of HR dependence. For example, ≥30% of patients develop metastatic, castration-resistant PCa (AR-transcript-resistant ARt) tumors [3] and 15–30% of BCa patients that develop endocrine resistance have tumors with reduced or lost ERα accumulation [1, 4]. In addition, at the time of diagnosis, 15–20% of BCa patients are innately ERα– (Triple Negative BCa) [5] and 10–20% of PCa patients are innately AR– (Small Cell Neuroendocrine PCa) [6]. Thus, there is a need to identify alternative therapeutic targets to ERα and AR hormone receptors.

Interleukin-1 (IL-1) is an inflammatory cytokine present in tumors that promotes tumor angiogenesis and metastasis [7]. IL-1 is elevated in BCa and PCA tumors [8–13] and correlates with low or lost ERα or AR accumulation [10, 14–17]. We discovered that IL-1 downregulates ERα and AR levels in HR+ BCa and PCa cell lines concomitant with the upregulation of pro-survival proteins that are basally high in HR+ cell lines [18, 19]. Thus, IL-1 may select for and promote the evolution of treatment-resistant cells, and our findings provide an opportunity to discover novel therapeutic targets for HR-dependent BCa and PCa.

We previously used RNA sequencing (RNA-seq) to identify genes that are modulated in response to the IL-1 family member, IL-1β, in the androgen-dependent AR+ PCA cell line, LNCaP [18]. Here, we performed RNA-seq for the estrogen-dependent ERα+ BCa cell line, MCF7, to identify genes that are modulated in response to both major IL-1 family members, IL-1α and IL-1β. We identified 350 genes that are conserved among IL-1-treated LNCaP and MCF7 cell lines that show similar expression patterns in untreated hormone-independent AR- PCa and MDA-MB-231 BCa cell lines. Not surprisingly, canonical pathway analysis reveals that the 350 gene set encodes for proteins that activate inflammatory signaling.

As proof-of-principal that the 350 gene set encodes for pro-survival genes, we selected two genes from the 350 gene set, Sequestome-1 (SQSTM1/p62; hereinafter, p62) and SRY (Sex-Determining Region Y)-Box 9 (SOX9), for functional analysis. RNA sequencing reveals that p62 and SOX9 are induced by IL-1 in LNCaP and MCF7 cells and are basally high in PC3 and MDA-MB-231 cells. p62 [20–32] and SOX9 [33–39] are overexpressed in both PCa and BCa patient tumor tissue, correlate with disease progression and treatment resistance, and support BCa and PCA tumor growth in vivo, indicating that these proteins are functional in cancer and clinically relevant. p62 is a multi-functional scaffold protein with well-characterized roles in autophagy and antioxidant response [40]. p62 sequesters cytotoxic protein aggregates, damaged organelles, and microbes into the autophagosome for degradation and biomolecule recycling [40–46], binds and poly-ubiquitinates Tumor Necrosis Factor Receptor-Associated Factor 6 (TRAF6), leading to the downstream activation of the pro- and anti-inflammatory transcription factor, Nuclear Factor Kappa Light Chain Enhancer of Activated B Cells (NFκB) [47, 48], and competitively binds Kelch-Like ECH-Associated Protein 1 (KEAP1) to promote activation of the antioxidant transcription factor, Nuclear Factor (Erythroid-Derived-2)-Like 2 (NRF2) [49–51]. SOX9 is a transcription factor with many diverse functions in development [52]. For example, SOX9 promotes epithelial-to-mesenchymal (EMT) transition of neural crest [53] and endocardial endothelial [54] cells during central nervous system and cardiac development, respectively, and induces Sertoli cell differentiation during testis development [55]. Thus, the functions of p62 and SOX9 in normal cell homeostasis and development provide cancer cells with a growth advantage and promote tumorigenicity.

We show that p62 and SOX9 are required for cell survival of HR- BCa and PCa cell lines, suggesting that HR- BCa and PCa cells evolve a survival requirement for p62 and SOX9. Interestingly, while IL-1 exposure elicits p62 and SOX9 induction concomitant with HR repression in HR+ BCa and PCa cell lines, down regulation of p62 or SOX9 had little or no effect on cell viability. Thus, p62 and SOX9 may play other pro-tumorigenic roles in response to IL-1 signaling and other genes identified in our signature may promote cell survival in response to IL-1-induced hormone receptor loss. We propose that IL-1 present in the inflammatory tumor microenvironment selects for hormone receptor-independent cells that are, consequently, resistant to hormone receptor-targeting therapy. Therefore, by identifying the conserved gene expression profile shared between HR+ BCa and PCa cell lines that lose hormone receptor accumulation in response to IL-1 and HR- BCa and PCa cell lines that are intrinsically hormone receptor-independent, we have identified putative therapeutic targets alternative to hormone receptors.

Methods

Cell culture

MCF7 (American Tissue Culture Collection (ATCC), Manassas, VA; HTB-22) and MDA-MB-231 (ATCC, Manassas, VA; HTB-26) BCa cell lines and LNCaP (ATCC, Manassas, VA; CRL-1740), PC3 (ATCC, Manassas, VA; CRL-1435), and DU145 (ATCC, Manassas, VA; HTB-81) PCa cell lines, were grown in a 37 °C, 5.0% (vol/vol) CO2 incubator in Dulbecco Modified Eagle Medium (DMEM;
Gibco, Waltham, MA; 1185–076) supplemented with 10% FB Essence (Seradigm, Radnor, PA; 3100–500), 0.4 mM of L-glutamine (L-glut; Gibco/Invitrogen, Waltham, MA; 25030081), and 10 U/ml of penicillin G sodium and 10 mg/ml of streptomyacin sulfate (pen-strep; Gibco/Invitrogen, Waltham, MA; 15140122). BT549 (ATCC, Manassas, VA; HTB-122) BCa cell line was grown in RPMI-1640 medium (Hyclone, Marlborough, MA; SH30027.01) supplemented with 10% FB essence, L-glut, and pen-strep.

**Cytokine treatment**

Human recombinant IL-1α (GoldBio, St Louis MO; 1110-01A-100) and IL-1β (GoldBio, St Louis MO; 1110-01B-100) were resuspended in 0.1% bovine serum albumin (BSA, Thermo Fisher Scientific; BP 1600–1) in 1X phosphate buffered saline (PBS). Cells were treated with 25 ng/ml of IL-1 or vehicle control (0.1% BSA in 1X PBS) added to DMEM or RPMI growth medium for the length of time indicated in the text.

**RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR)**

RNA was extracted from cells treated with cytokines using GeneJET RNA Purification kit (Thermo Fisher Scientific, Waltham, MA; K0732) as per manufacturer’s instructions. Genomic DNA contamination was removed by treating 1 μg of RNA with 1 U of DNase-I (Thermo scientific, Waltham, MA; EN05 21) following manufacturer’s instructions. Complementary DNA was synthesized using iScript Reverse Transcription Supermix (Bio-Rad, Hercules, CA; 170–8841). RT-qPCR reactions were performed using the iTaq Universal SYBR Green Supermix (Bio-Rad, Hercules, CA; 1708841). The quantity trimmed using fastq-mcf (ea-utils/1.1.2–806) [58]. Trimmed fastq files were mapped to hg19 (UCSC version from igenomes) using TopHat [59], duplicates were marked using picard-tools (v1.127 https://broadinstitute.github.io/picard/), read counts were generated using featureCounts [60] and differential expression analysis was performed using edgeR [61]. Differential gene expression lists were generated using the following cut-offs: log2ounts per million (CPM) ≥ 0, log2 fold change (FC) ≥ 0.6 or ≤ −0.6, false discovery rate (FDR) ≤ 0.05. Pathway analysis was conducted using QIAGEN’s Ingenuity Pathway Analysis tool (http://www.qiagen.com/ingenuity) or using the web-based gene ontology tool GOrilla (http://ceb-gorilla.cs.technion.ac.il/). RNA-seq datasets generated for this study are available at GEO NCBI, accession GSE136420.

**Western blot and antibodies**

Protein was isolated from cells using NP40 lysis buffer (0.5% NP40 [US Biological, Salem, MA; N3500], 50 mM of Tris [pH7.5], 150 mM of NaCl, 3 mM of MgCl2, 1X protease inhibitors [Roche, Mannheim, Germany; 05892953001]). Protein concentration was measured using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA; 23,227). For Western blot analysis, equal protein concentrations were loaded onto and separated in 12% (wt/vol) sodium dodecyl sulfate polyacrylamide gel (40% acrylamide/bisacrylamide solution, Bio-Rad, Hercules, CA; 172–5125) as per manufacturer’s instructions and the Bio-Rad CFX Connect. The cycle times for each gene was normalized to β-actin. Relative mRNA levels were calculated using 2^−ΔΔCT method. 5’ to 3’ primer sequences: Custom primers were obtained from Sigma-Aldrich, St. Louis, MO. CCL20 forward, GAGTTTGCTCTGGCTGCTTT; CCL20 reverse, AA AGTTGTCTTGTCTTCTGAT; CDK2 forward, CGA GCTCTGAAATCCTCCTG; CDK2 reverse, GCGAG TCACCATCTCAGCAA; CD68 forward, CAGGGAAAT GACTGTCTCCTACA; CD68 reverse, CAGTTGCCCTC TGTAACCCCTGG; CXCR7 forward, ACGTCTGCGGT CCAAACATGTA; CXCR7 reverse, AAGCCCAAGACA ACGGAGAC; IL-8 forward, ACACTGGCACCACA CAGAAAAT; IL-8 reverse, AACTTCTCCACAAAACT CTTG; MMP16 reverse, TCAGCAGTCGGAGCCCGTT TTG; MMP16 reverse, AAATACTGCTCCGTTCCG; PLK1 forward, TGTACCAGAGGATGACCTGC; PLK1 reverse, TGTACCAGAGGATGACCTGC; p62 forward, AAA TGGGTCCACCAAGAAAACCTGGA; p62 reverse, TCA ACTTCAATGGCCAGAGGGCTA; β-actin forward, GATGAGATGGCATTGCTTT; β-actin reverse, CACTTCAGGGTGCTCATTTT.

**RNA sequencing (RNA-seq) analysis**

RNA-seq was performed by the Genome Center at the University of Texas at Dallas (Richardson, TX). Total RNA library was prepared using Illumina Truseq Stranded Total RNA prep Gold kit (Illumina). The prepared libraries were sequenced on an Illumina NextSeq 500 platform (San Diego, CA) with 75 bp single-end reads. Fastq files were checked for quality using fastqc (v0.11.2) [56] and fastq_screen (v0.4.4) [57] and were quality trimmed using fastq-mcf (ea-utils/1.1.2–806) [58]. Trimmed fastq files were mapped to hg19 (UCSC version from igenomes) using TopHat [59], duplicates were marked using picard-tools (v1.127 https://broadinstitute.github.io/picard/), read counts were generated using featureCounts [60] and differential expression analysis was performed using edgeR [61]. Differential gene expression lists were generated using the following cut-offs: log2 counts per million (CPM) ≥ 0, log2 fold change (FC) ≥ 0.6 or ≤ −0.6, false discovery rate (FDR) ≤ 0.05.

**GO and pathway analysis**

Pathway analysis was performed using pathway analysis tool (http://www.qiagen.com/ingenuity) or using the web-based gene ontology tool GOrilla (http://ceb-gorilla.cs.technion.ac.il/). RNA-seq datasets generated for this study are available at GEO NCBI, accession GSE136420.
were visualized using Clarity Western ECL Substrate (Bio-Rad, Hercules, CA; 1,705,061) and imaged using Amersham Imager 600 (GE, Marlborough, MA). **Primary antibodies:** AR (Cell Signaling Technology, Danvers, MA; 5153S), ERα (Cell Signaling Technology, Danvers, MA; 8644), p62 (Abcam, Cambridge, MA; H00008878-M01), SOX9 (Cell Signaling, Danvers, MA; 82630S), β-actin (Santa Cruz Biotechnology, Dallas, TX; sc-69,879). **Secondary antibodies:** Sheep anti-mouse (Jackson ImmunoResearch Laboratories, West Grove, PA; 515–035-062), goat anti-rabbit (Sigma-Aldrich, St. Louis, MO; A6154).

**Small interfering RNA (siRNA) and drug treatments**

siRNA: Cells were transfected with a pool of four unique p62 siRNA duplexes (Dharmacon, Lafayette, CO; M-010230-00-0020) or SOX9 siRNA duplexes (Dharmacon, Lafayette, CO; M-021507-00-0010) using siTran 1.0 transfection reagent (Origene, Rockville, MD; TT300001). Non-targeting siRNA duplex was used as a negative control (Dharmacon, Lafayette, CO; D-001210-02-20). RT-qPCR was used to confirm mRNA knock-down. 

**Drug treatment:** Verteporfin (Sigma-Aldrich, St. Louis, MO; SML0534) was resuspended in DMSO. Cells were exposed to 10 μM verteporfin or DMSO and western blotting or immunostaining for p62 oligomerization was performed to determine treatment efficacy.

**Cell counts**

Cells were fixed with cold 100% methanol for 15 min. The nuclei were then stained with DAPI (Roche Diagnostics, Basel, Switzerland; 10,236,276,001) and counted on the Cytation3 Imaging Reader (BioTek, Winooski, VT).

**MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium bromide] assay**

MTT assay (Trevigen; 4890–25-K) was performed according to manufacturer’s instructions. Cell viability was quantified as the optical density (OD) read at wavelengths 540 nm and 650 nm. The final OD was calculated as follows: OD 540 nm – OD 640 nm. OD was measured using the Cytation3 Imaging Reader (BioTek, Winooski, VT).

**Immunostaining**

Cells were fixed and permeabilized with 100% methanol at −20 °C for 30 min. Fixed cells were blocked with 2.5% BSA in 1X PBS at room temperature for at least 30 min. Antibodies were diluted in 2.5% BSA in 1X PBS. Cells were incubated in primary antibody overnight at 4 °C, washed with 1X PBS, and then incubated with fluorescently labeled secondary antibody overnight at 4 °C in the dark. Primary antibody: p62 (Santa Cruz Biotechnology, Dallas, TX; sc-28,359). Fluorescently labeled secondary antibody: Alexa Fluor 488, goat anti-mouse (Invitrogen, Waltham, MA; A11001). Nuclei were stained with DAPI (Roche Diagnostics, Basel, Switzerland; 10,236,276,001). Immunostained cells were imaged at 40X magnification using a Nikon epifluorescence microscope (Nikon, Melville, NY). Densitometry was performed using Image J (https://imagej.nih.gov/ij/). β-actin is the western blot loading control and the protein/β-actin ratio is normalized to treatment control for densitometry.

**Statistical analysis**

Statistical significance was determined using unpaired student t-test. P-value ≤0.05 is considered statistically significant. Graphs are shown as the average of a minimum of n = 3 biological replicates ± standard deviation (STDEV).

**Results**

**Identification of an IL-1 conferred gene signature in hormone receptor positive BCa and PCa cell lines that mimics a basal gene expression pattern in hormone receptor negative BCa and PCa cells**

We previously found that IL-1 represses hormone receptors in ERα+/PR+ BCa [19] and AR+ PCa [18, 62] cell lines comonitant with p62 upregulation, while ERα−/PR− BCa and AR− PCa cell lines intrinsically have high basal p62. This led us to speculate that IL-1 elicits similar changes in gene expression in hormone receptor positive (HR+) BCa and PCa cells that mimic intrinsic gene expression patterns in hormone receptor negative (HR−) BCa and PCa cells. Such changes would enable BCa and PCa cells to elicit compensatory survival pathways in the absence of hormone receptor activity and, thus, these changes in gene expression could confer resistance to hormone therapy for BCa and PCa tumor cells. To identify the conserved gene signature conferred by IL-1 in HR+ BCa and PCa cells that mimics intrinsic gene expression patterns in HR− BCa and PCa cells, we performed RNA sequencing (RNA-seq) followed by differential gene expression analysis (log 2 CPM ≥ 0, log 2 FC ≥ 0.6 or ≤ −0.6, and FDR ≤ 0.05) for IL-1α- or IL-1β-treated ERα+/PR+ MCF7 BCa cell line, IL-1β-treated AR− LNCaP PCa cell line, vehicle control-treated ERα−/PR− MDA-MB-231 BCa cell line, and vehicle control-treated AR− PC3 PCa cell line. LNCaP and PC3 RNA-seq data was previously reported [18] (GSE105088). For BCa cell line RNA-seq, RNA was isolated from MCF7 cells treated with vehicle control, 25 ng/ml IL-1α, or 25 ng/ml IL-1β for 5 days and MDA-MB-231 cells were treated with vehicle control only. For PCa cell line RNA-seq, as previously reported, RNA was isolated from LNCaP cells treated with vehicle control or 25 ng/ml IL-1β for 3 days and PC3 cells were treated with vehicle control only. Five sets of differential gene expression analysis were performed: (Set 1) MCF7 cells treated with
vehicle control versus IL-1α ("MCF7_VEH-VS-MCF7_IL1A"); (Set 2) MCF7 cells treated with vehicle control versus IL-1β ("MCF7_VEH-VS-MCF7_IL1B"); (Set 3) MCF7 cells treated with vehicle control versus MDA-MB-231 cells treated with vehicle control ("MCF7_VEH-VS-231_VEH"); (Set 4) LNCaP cells treated with vehicle control versus IL-1β ("LNCaP_VEH-VS-LNCaP_IL1B"); and LNCaP cells treated with vehicle control versus PC3 cells treated with vehicle control ("LNCaP_VEH-VS-PC3_VEH") (Fig. 1a). We identified 2735 genes in the intersection of Set 1, 2, and 3 and of those genes, 1707 are consistent in fold change direction (Set 6). Set 6 are the genes that are induced or repressed by both IL-1α and IL-1β in MCF7 cells which are, respectively, basally high or low in MDA-MB-231 cells. We identified 2786 genes in the intersection of Set 4 and 5 and of those genes, 1900 are consistent in fold change direction (Set 7). Set 7 are the genes that are induced or repressed by IL-1β in LNCaP cells which are, respectively, basally high or low in PC3 cells. Finally, we identified the 420 genes in the intersection of Set 6 and Set 7 and of those genes, 350 are consistent in fold change direction (Set 8; Additional file 3: Table S1). Set 8 are the genes that are induced or repressed by IL-1 in MCF7 and LNCaP cells which are, respectively, basally high or low in MDA-MB-231 and PC3 cells. Thus, the 350 gene set represents conserved genes in both BCa and PCa cells that are expected to promote cell survival and tumorigenicity when hormone receptor signaling is lost.

Validation of select genes from the 350 gene signature

We arbitrarily selected six upregulated genes (CCL20, CD68, IL-8, p62, SOX9, Zeb1) and four downregulated genes (CDK2, CXCR7, MMP16, PLK1) from our 350 gene set to validate by RT-qPCR. RNA-seq was performed for HR+ MCF7 and LNCaP cells treated with 25 ng/ml
IL-1 and for untreated HR− MDA-MD-231 and PC3 cells; therefore, MCF7 and LNCaP cells were treated with 25 ng/ml IL-1α or IL-1β and MDA-MB-231 and PC3 cells were treated with vehicle control for RT-qPCR analysis. In addition to MDA-MB-231 and PC3 cell lines, we also performed RT-qPCR for basal gene expression in an additional ERα+/PR− BCa cell line, BT549, and an additional AR− PCa cell line, DU145.

RT-qPCR confirmed that IL-1 induces CCL20, IL-8, p62, and Zeb1 in MCF7 (Fig. 2a) and LNCaP (Fig. 2b) cells. We detected a significant increase in CD68 and SOX9 mRNA (Fig. 2b) and/or protein (Fig. 3a) in IL-1-treated LNCaP cells. We did not detect an increase in SOX9 mRNA in IL-1-treated MCF7 cells (Fig. 2a) and we detected only a slight increase in SOX9 protein (Fig. 3a). CD68 mRNA levels were only slightly induced by IL-1β in MCF7 cells (Fig. 2a). IL-1-induced fold changes detected by RNA-seq for CD68 and SOX9 were much greater in LNCaP cells (CD68 \( \log_2 FC = 4.46 \); SOX9 \( \log_2 FC = 4.33 \) ) than in MCF7 cells (CD68 \( \log_2 FC = 0.89 \) (IL-1α), 0.62 (IL-1β); SOX9 \( \log_2 FC = 1.31 \) (IL-1α), 1.36 (IL-1β)) (Additional file 3: Table S1), which may correspond with our inability to detect induction for these genes by RT-qPCR in MCF7 cells. Finally, RT-qPCR confirmed that IL-1α and IL-1β repress CDK2, CXCR7, MMP16, and PLK1 in MCF7 and LNCaP cells (Fig. 2).

RT-qPCR confirmed that CCL20, CD68, IL-8, p62, and Zeb1 are basally high MDA-MB-231, BT549, PC3, and DU145 cells (Fig. 2). RT-qPCR and western blot confirmed that SOX9 mRNA (Fig. 2) and protein (Fig. 3a) are basally high in MDA-MB-231 and PC3 cells and SOX9 protein is basally high in BT549 (Fig. 3a). However, SOX9 mRNA is not basally high in BT549 or DU145 (Fig. 2). RT-qPCR confirmed that CXCR7 is basally low in MDA-MB-231, BT549, PC3, and DU145 cells, MMP16 is basally low in MDA-MB-231, PC3, and DU145 cell lines, and CDK2 and PLK1 are basally low in BT549 cells (Fig. 2). However, inconsistencies with RNA-seq, CDK2 and PLK1 are not basally low in MDA-MB-231, PC3, and DU145 cells by RT-qPCR and MMP16 is basally high in BT549 (Fig. 2). Cell line differences among the HR− cell lines likely reflect the inconsistencies we observed for BT549 and DU145 cell lines, which were not sequenced. In addition, RNA sequencing can be more sensitive than RT-qPCR analysis of gene expression, which may also account for inconsistency. For example, MDA-MB-231 and PC3 RNA sequencing show much lower basal expression for CXCR7 (\( \log_2 FC = -7.61 \) and −9.02) and MMP16 (\( \log_2 FC = -2.33 \) and −2.70) than for CDK2 (\( \log_2 FC = -0.86 \) and −1.29) and PLK1 (\( \log_2 FC = -0.22 \) and −1.43) (Additional file 3: Table S1).

Taken together, of the 10 arbitrary genes we picked for validation of the LNCaP, MCF7, PC3 and MDA-MB-231 RNA-seq results, 10 genes were validated by RT-qPCR in LNCaP cells, 8 genes were validated in MCF7, MDA-MB-231, BT549, and PC3 cells, and 7 genes were validated in DU145 cells. Taken together, our RT-qPCR of arbitrarily selected genes provides additional evidence of the 350 gene set expression patterns identified in MCF7, LNCaP, MDA-MB-231, and PC3 cells by RNA-seq.

**p62 and SOX9 are upregulated in hormone receptor-independent LNCaP and MCF7 cells**

Given that IL-1 reduces hormone receptors concomitant with the upregulation of pro-survival and pro-tumorigenic genes, such as p62 [20, 23, 24, 29, 31, 62, 63], we hypothesized that IL-1 can promote resistance to hormone receptor-targeted therapy. Enzalutamide is an anti-androgen therapeutic that inhibits AR activity and fulvestrant is an anti-estrogen therapeutic that inhibits ERα activity. Enzalutamide [64] and fulvestrant [65] are used to treat AR+ PCa and ERα+ BCa patients, respectively. However, patients can develop treatment resistance. Therefore, labs have produced publicly available data sets investigating changes in gene expression patterns in treatment-resistant cells to better understand resistance mechanisms and identify alternative therapeutic targets.

We compared our 350 gene set to published RNA-seq data from enzalutamide-resistant LNCaP3 sublines (GSE99381; APIPC subline versus APIPC_P (parental), Additional file 3: Table S1) or fulvestrant-resistant MCF7 [66] sublines (GSE74391; ICI182R1 or ICI182R6 subline versus fulvestrant-sensitive subline (parental), Additional file 3: Table S1). Among the selected upregulated genes we chose for RT-qPCR confirmation, p62 and SOX9 were also upregulated in both treatment-resistant subline models (Additional file 3: Table S1). Downregulation of AR or ERα/PR and target gene expression (e.g. AR target gene, KLK3) in the LNCaP3 or MCF7 [66] treatment-resistant sublines (Additional file 3: Table S1) indicates these models evolved to survive without hormone receptor activity. Thus, p62 or SOX9 may promote survival in BCa and PCa cells that lose hormone receptor accumulation in response to IL-1, as well as promote survival in BCa and PCa cells that intrinsically lack hormone receptors.

**p62 and SOX9 are cytoprotective for HR− BCa cell lines**

To determine if p62 or SOX9 are required for viability in hormone receptor-independent cells, we siRNA-silenced p62 or SOX9 in IL-1-treated HR− BCa and PCa cell lines and in HR− cell lines that intrinsically lack hormone receptor accumulation. AR− LNCaP PCa cells and ERα− MCF7 BCa cells were first transfected with p62 or SOX9 siRNA and the following day, treated with vehicle control, 25 ng/ml IL-1α, or 25 ng/ml IL-1β for 3 days (LNCaP, MCF7) or 5 days (MCF7). siRNA efficacy was confirmed by RT-qPCR or western blot and cells were assayed for viability using MTT (Additional file 1: Figure S1). siRNA-mediated
Fig. 2 RT-qPCR validation of select genes from 350 gene signature. RT-qPCR was performed for select genes for LNCaP and MCF7 cell lines treated with vehicle control or 25 ng/ml IL-1α or IL-1β for 3 days (LNCaP) or 5 days (MCF7). MDA-MB-231, BT549, PC3 and DU145 cell lines were treated with vehicle control only. RT-qPCR is shown for the BCa (a) and PCa (b) cell lines. RT-qPCR shows that CCL20, CD68, IL8, p62, SOX9 and Zeb1 are induced by IL-1 in MCF7 and/or LNCaP cell lines and are basally high in MDA-MB-231, BT549, PC3 and/or DU145 cell lines. RT-qPCR shows that CXCR7 and MMP16, but not CDK2 or PLK1, are downregulated by IL-1 in MCF7 and/or LNCaP cell lines and are basally high in MDA-MB-231, BT549, PC3 and/or DU145 cell lines. N = 3 biological replicates; error bars, +/−STDEV; p-value, * ≤ 0.05, ** ≤ 0.005, *** ≤ 0.0005; mRNA fold change is normalized to MCF7 vehicle control for the BCa cell lines and to LNCaP vehicle control for the PCa cell lines.
Fig. 3 (See legend on next page.)
loss of p62 or SOX9 reduced LNCaP viability 10–25% in the presence of IL-1α or IL-1β, but had no effect on cell viability under control growth conditions (Additional file 1: Figure S1A). p62 or SOX9 siRNA had no effect on MCF7 viability in response to IL-1, nor under control growth conditions (Additional file 1: Figure S1B & C).

While p62 or SOX9 down regulation had little to no effect on cell viability in IL-1-treated LNCaP and MCF7 cells, repression of basal p62 or SOX9 was significantly cytotoxic for HR− BCa and PCa cell lines. MDA-MB-231, BT549, PC3, and DU145 HR+ cells were transfected with p62 or SOX9 siRNA (Fig. 3b) and cell viability was determined on day 1 or day 3 using MTT (Fig. 3c) or by recording cell number on day 1, 2, and 3 (Fig. 3d). The loss of basal p62 or SOX9 reduced cell viability 20–60% in HR− BCa and PCa cell lines (Fig. 3c) and caused a > 30% reduction in cell number by day 3 (Fig. 3d).

Taken together, HR− BCa and PCa cell lines have evolved a survival requirement for basal p62 or SOX9 function, while HR+ BCa and PCa cell lines are less dependent on p62 or SOX9 for survival. Thus, p62 and SOX9 may be upregulated by IL-1 in HR+ BCa and PCa cells to mediate other HR+ tumorigenic functions. In kind, other genes in the 350 gene list may encode for cytoprotective proteins that protect against IL-1-induced hormone receptor repression.

Verteporfin is cytotoxic for HR+ BCa and PCa cell lines

BCa or PCa cells that acquire hormone receptor independence or intrinsically lack hormone receptor activity are not susceptible to hormone receptor-targeting drugs, such as enzalutamide or fulvestrant. Therefore, alternative therapeutic targets are needed. The p62 inhibitor, verteporfin (Visudyne®), is an FDA-approved, photosensitizing drug used with laser light to treat leaky blood vessels in the eye caused by macular degeneration. Currently, verteporfin is being tested in a Phase 1 clinical trial as a photosensitizer for the SpectraCure P18 photodynamic therapy system for recurrent PCa (NCT03067051). Recently, verteporfin, alone, was shown to reduce the tumor growth of subcutaneous prostate epithelial xenografts overexpressing p62 and verteporfin was able to reduce the growth of PC3 xenografts [20]. Verteporfin oligomerizes p62 (Fig. 4a & b; Additional file 2: Figure S2A), thereby, preventing p62 interaction with binding partners and inhibiting p62 function [20, 67]. Given that the HR− BCa and PCa cell lines demonstrated significant dependency on p62 for survival, we treated HR− BCa and PCa cell lines with 2.5, 5, or 10 μM verteporfin for 3 days and assayed cells for viability using MTT (Additional file 2: Fig. 2b). HR− BCa cells were also treated with 10 μM verteporfin for 5 days and assayed cell viability using MTT (Fig. 4c) or by recording cell number on day 1, 3, and 5 (Fig. 4d). Verteporfin is cytotoxic for HR− BCa and PCa cell lines and, thus, may be a rationale therapeutic alternative.

The 350 gene signature maps to pro-tumorigenic processes in BCa and PCa cells

We used the IPA Canonical Pathways module to identify signaling pathways represented in our 350 gene set and found that the gene set encodes for proteins that are predicted to activate inflammatory signaling, including interferon (z-score = 3.2, -log p-value = 9.86E+00), IL-1 (z-score = 2, -log p-value = 1.26E+00), and IL-8 signaling (z-score = 1.7, -log p-value = 4.10E+00) (Additional file 3: Table S1). In addition to IPA, we also used the web-based gene ontology tool, GOrilla (http://cbl-gorilla.cs.technion.ac.il/) to perform gene enrichment analysis for biological processes represented in the 350 gene set. Similar to IPA, GOrilla also reports that the 350 gene set is enriched in genes that encode for proteins involved in inflammatory signaling including defense response (enrichment score = 3.83, p-value = 8.94E-21), immune system process (enrichment score = 2.55, p-value = 8.42E-19), and interferon signaling (enrichment score = 14.23, p-value = 7.92E-17) (Additional file 3: Table S1).

We also used the IPA Regulator Effects module to predict cancer-specific networks encoded by our 350 gene set and we selected the networks in which both p62 and SOX9 were target molecules. We found three such networks in which upstream regulators CTNNB1 (z-score 2.738, p-value 5.12E-07), FGF2 (z-score 2.091, p-value 2.21E-09) and TNF (z-score 7.192, p-value 4.87E-28) were found to be activated and predicted to promote neoplasia of cells (z-score 2.699, p-value 5.20E-12 (CTNNB1)) and malignancy (z-score 2.251, p-value 1.41E-13 (FGF2, TNF)) (Additional file 3: Table S1). Thus, the IL-1-conferred 350 gene set encodes multiple different pro-tumorigenic...
signaling pathways conserved among multiple different regulators.

**Predicted p62 and SOX9 target molecules and functional networks in the 350 gene signature**

Finally, given that siRNA-mediated loss of p62 or SOX9 are cytotoxic for HR− BCa and PCa cells, we used the IPA Upstream Regulators module to identify p62 or SOX9 predicted target molecules and functional networks in the 350 gene data set. p62 is predicted to induce CXCL2, IL15RA, IRF1, PLAT, RGCC, and RSAD2 expression (z-score = 2.449, p-value = 8.38E-05) (Fig. 5a; Additional file 3: Table S1) and predicted to activate HIF1A, NFkB1, NFKBIA, NFKB (complex), RELA, and SQSTM1(p62) signaling (Fig. 5c; Additional file 3: Table S1). p62 is also predicted to function upstream of active IL-1β signaling and upstream of attenuated NR3C1 function; but IPA analysis of the 350 gene set did not predict if p62 promotes or prevents the activation of IL-1β signaling or the inhibition NR3C1 function (Fig. 5c). Notably, we show that IL-1 induces p62 expression and, as stated earlier, p62 binds and poly-ubiquitinates TRAF6, leading to NFKB transactivation [47, 48]. NFKB is the canonical mediator of IL-1 inflammatory signaling [68] and IL-1β is an NFKB target gene [69].
Thus, p62 functions in a positive feedback loop to induce IL-1β production and signaling. NR3C1 encodes the glucocorticoid nuclear receptor which represses inflammatory gene expression, including IL-1-regulated genes [70]. Thus, p62 is expected to participate in the cross-talk between IL-1 and glucocorticoid signaling and, in turn, promote NR3C1 inhibition. In kind, the p62-regulated genes and interactive networks are known mediators of inflammatory signaling and immunity (CXCL2, IL15RA, IRF1, PLAT, RGCC, and RSAD2), as well as hypoxia (HIF1A), fibrinolysis (PLAT), and cell cycle regulation (RGCC). SOX9 did not appear as a master regulator in IPA; therefore, we manually extracted its interactome from IPA; overlapped the results with the 350 gene list to identify relevant interactions. SOX9 is predicted to activate VNN1 expression and mediate FN1 and T-Cell Factor (TCF) signaling.

### Discussion

IL-1 conferred 350-gene signature is enriched in genes and pathways that promote BCa and PCa cell survival and disease progression

We have previously shown that HR\(^{\text{low}}\) BCa [19] and PCa [18, 62] cell populations are enriched when HR\(^{+}\) cells are exposed to IL-1. Here, we sought to compare the gene expression pattern overlap between cells that lose hormone receptors in response to IL-1 to that of cells that are intrinsically HR\(^{-}\). We identified 1707 genes in BCa cells and 1900 genes in PCa cells that are upregulated by IL-1 in HR\(^{+}\) cells and are basally high in HR\(^{-}\) cells or are downregulated by IL-1 in HR\(^{+}\) cells and are basally low in HR\(^{-}\) cells. To identify genes that were common to both BCa and PCa, we looked for overlapping genes and filtered our list down to a set of 350 genes. This conserved expression pattern represents IL-1-induced signaling in HR\(^{+}\) BCa and PCa cells and constitutive signaling in HR\(^{-}\) BCa and PCa cells.

To gain insight in the functional output of the 350 gene set, we used IPA and GOrialla to predict activation and enrichment of canonical pathways. IL-1 is an inflammatory cytokine, therefore, as expected, the 350...
gene set encodes for activation and enrichment of inflammatory pathways like IL-1, IL-8, IL-6, interferon, and TNF receptor signaling. Importantly, inflammatory signaling promotes BCa [9] and PCa [71] progression. We also used IPA to identify cancer-specific networks encoded by the 350 gene set that also include p62 and SOX9 as downstream target signaling molecules and found that IL-1 is predicted to activate molecular programs similar to CTNNB1, Fibroblast Growth Factor (FGF2), and Tumor Necrosis Factor (TNF). CTNNB1 encodes for beta-catenin (β-catenin) and mediates the canonical WNT pathway regulation of cell fate and tissue development [72]. WNT-β-catenin signaling promotes tumor progression, stem cell proliferation and treatment resistance in BCa [73] and PCa [74]. FGF2 signaling regulates normal tissue development, angiogenesis, and wound healing [75] and FGF2 promotes tumor progression, metastasis, and/or angiogenesis in BCa [76] and PCa [77]. Finally, TNF is an inflammatory cytokine that promotes BCa EMT, metastasis, and invasion [78] and promotes PCa androgen independence, EMT, metastasis, and invasion [79]. Taken together, the IL-1-conferred 350 gene signature encodes for proteins that have been shown to participate in conserved pro-tumorigenic pathways in response to multiple different stimuli.

While BCa and PCa cells that lose hormone receptor accumulation in response to IL-1 signaling can elicit pro-survival pathways, IL-1 signaling activates other biological processes in BCa [12, 80–82] and PCa [10, 83–89] cells that promote tumorigenicity, such as treatment resistance, angiogenesis, differentiation, EMT, metastasis, and invasion. Thus, given that we found that p62 or SOX9 is not required for the viability of IL-1-treated HR+ BCa and/or PCa cells, p62 and SOX9 likely function in these other IL-1-regulated pro-tumorigenic pathways. Therefore, targeting p62 or SOX9 in BCa and PCa cells that acquire inflammation-induced hormone receptor independence could prevent tumor growth or metastasis of these treatment-resistant cells.

**p62 and SOX9 are rational therapeutic targets for BCa and PCa when hormone receptor signaling is lost**

p62 protein is overexpressed in PCa patient tumors, is prognostic, and correlates with advanced PCa disease [21, 32], and p62 protein accumulation is elevated in BCa patient tumors relative to the normal adjacent tissue [24]. Furthermore, SOX9 was found to be highly expressed in BCa patient tumors relative to normal tissue and showed strongest expression in ERα+ tumors [39], and elevated SOX9 levels correlate with disease progression in PCa [34]. Analysis of publicly available gene expression data from ERα−, fulvestrant-resistant MCF7 and AR−, enzalutamide-resistant LNCaP sublines showed that p62 and SOX9 are upregulated. Fulvestrant and enzalutamide, respectively, block activity of ERα and AR, thus HR+ BCa and PCa are intrinsically resistant to these HR-targeting drugs. We found that gene silencing p62 or SOX9 in HR− BCa and PCa cell lines is cytotoxic, including treatment with the p62-targeting drug, verteporfin. Thus, cells that evolve HR-independence and treatment resistance could concomitantly evolve dependency on cytotoxic, pro-tumorigenic p62 or SOX9, making p62 and SOX9 rational therapeutic targets in hormone receptor-independent BCa and PCa.

IPA predicts p62 to be a master regulator of inflammation, immunity, hypoxia, fibrinolysis, and the cell cycle and predicts SOX9 to function in immunity and WNT signaling through their respective interactions within the 350 gene set. For example, p62-NRF2 signaling was shown to attenuate reactive oxygen, maintain stemness and promote tumor growth of BCa cells [28] and SOX9 was shown to activate WNT signaling and drive WNT-mediated PCa tumor growth [90]. Thus, the IPA-predicted cellular functions of p62 and SOX9 in the context of the 350 gene set provides insight into how p62 and SOX9 promote BCa and PCa survival and, in particular, promote cell survival or tumorigenicity when HR
signaling is lost. Equally important, in addition to p62 and SOX9, our 350 gene set provides a myriad of additional rationel target molecules and networks conserved among both hormone receptor-independent, treatment-resistant BCa and PCa and, thus, our 350 gene set has the potential to have a broader patient impact.

Conclusions
Having discovered that IL-1 represses hormone receptors in both ERα+ BCa and AR+ PCa cell lines, we identified a conserved gene expression signature between IL-1-treated HR+ BCa and PCa cell lines and untreated HR+ cell lines. We performed functional bioinformatics analyses to predict cellular function of the gene signature. This approach revealed potential therapeutic targets, p62 and SOX9, and identified signaling pathways downstream of inflammatory (e.g. IL-1 and TNF), FGF, or WNT signaling that could be targeted in both hormone receptor-independent, treatment-resistant BCa and PCa (Fig. 6).

Supplementary information
Supplementary information accompanies this paper at https://doi.org/10.1186/s12885-020-6529-9.

Additional file 1: Figure S1. p62 and/or SOX9 are not required for cell survival in HR+ PCa and BCa cell lines. (A) UNCAP and (B) MCF7 cell lines were treated with 70 nM control siRNA (“c”), p62 siRNA, or SOX9 siRNA and after 1 day in siRNA, cells were treated with vehicle control (“veh”) or 25 ng/ml IL-1 for an additional 3 days. Western blot was performed to confirm p62 or SOX9 knockdown and MTT was performed to determine cell viability. (C) MCF7 cell lines were treated with 70 nM control siRNA, p62 siRNA, or SOX9 siRNA and after 1 day in siRNA, cells were treated with vehicle control or 25 ng/ml IL-1 for an additional 5 days. siRNA is transient; therefore, an additional 70 nM siRNA was added on day 4 after the initial siRNA treatment. RT-qPCR was performed to validate p62 or SOX9 knockdown and MTT was performed to determine cell viability. Loss of p62 or SOX9 is slightly cytotoxic for IL-1-treated UNCAP but is not cytotoxic for IL-1-treated MCF7 cells. N = 3 biological replicates; error bars, +/-STDDEV; p-value, **≤0.05, *** ≤0.005. mRNA fold change and cell viability are normalized to control siRNA for each treatment. Western blot densitometry is shown.

Additional file 2: Figure S2. Verteporfin is cytotoxic for HR+ BCa and PCa cell lines. (A) MDA-MB-231, BT549, PC3, and DU145 cell lines were treated with vehicle control, 2.5, 5, or 10 μM verteporfin for 1 day. Western blot analysis shows oligomerized p62 (p62*), indicating treatment efficacy. (B) Cells were treated with vehicle control, 2.5, or 10 μM verteporfin for 3 days and MTT assay was performed to assess cell viability. Verteporfin is cytotoxic for MDA-MB-231, BT549, PC3, and DU145 cell lines. N = 3 biological replicates; error bars, +/-STDDEV; p-value, **≤0.005, *** ≤0.0005. Cell viability is normalized to vehicle control for each cell line.

Additional file 3: Table S1. Gene expression and ontology data. The “Table Legend” tab is a description of each worksheet tab. Each worksheet contains the gene expression data, IPA analysis, or GOriilla analysis described in the paper.

Abbreviations
AR: Androgen receptor; BCa: Breast cancer; CTNNB1: Beta catenin; CXCL2: C-X-C motif chemokine ligand 2; EMT: Epithelial-to-mesenchymal transition; ERα: Estrogen receptor alpha; FGF2: Fibroblast growth factor 2; FN1: Fibronectin 1; HIF1A: Hypoxia inducible factor 1 subunit alpha; HR: Hormone receptor; IL15RA: Interleukin 15 receptor subunit alpha; IL-1α: Interleukin-1 alpha; IL-1β: Interleukin-1 beta; IL-8: Interleukin-8; IPA: Ingenuity pathway analysis; IRF1: Interferon regulatory factor 1; KEP1: Kelch-like ECH-associated protein 1; NFKBIA: NFkB Inhibitor alpha; NFkB: Nuclear factor kappa light chain enhancer of activated b cells; NR3C1: Glucocorticoid receptor; NRF2: Nuclear factor (erythroid-derived 2)-like 2; PCa: Prostate cancer; PLAT: Tissue-type plasminogen activator; RELA: NFkB Transcription factor p65; RGC2: Regulator of cell cycle, RAD52: Radical S-adenosyl methionine domain containing 2; SOX9: SRY (Sex Determining Region Y)-Box 9; Sqstm1: Sequestome-1; TCF: T-cell factor; TNF: Tumor necrosis factor; TRAF6: Tumor necrosis factor receptor-associated factor 6; VNN1: Vanin 1

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Authors’ contributions
AF, experiment design, execution, analysis, manuscript preparation. MK, bioinformatics analysis, manuscript preparation. ST, optimization of PCa culture, RT-qPCR, western blot analysis, manuscript editing. HD, assisted with siRNA and cell viability experiments. KD and FB, analysis of publicly available expression data. MB, assisted with verteporfin treatments. VA, AW, R Meade, undergraduate student researchers assisted AF with experiments. R Mistry and NG, optimized PCa and BCa cell culture and treatment conditions for siRNA and cell viability. CX, head of bioinformatics core. ND, senior and corresponding author. All authors read and approved the final manuscript.

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Availability of data and materials
RNA-seq datasets generated for this study are available at GEO NCBI, accession GSE136420.

Ethics approval and consent to participate
All cell lines used in the study are commercially available and purchased from American Tissue Culture Collection (ATCC). We hereby confirm that none of the used cell lines require any ethics approval for their use.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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