EP300 Knockdown Abrogates Cancer Stem Cell Phenotype, Tumor Growth and Metastasis in Triple Negative Breast Cancer

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Alexander Ring
UniversitatsSpital Zurich
alexander.ring@usz.ch
ORCID: https://orcid.org/0000-0002-0092-1323

Pushpinder Kaur
Vanguard University of Southern California

Julie E. Lang
Vanguard University of Southern California

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Abstract

**Background:** Triple negative breast cancer (TNBC) is an aggressive breast cancer subtype with basal features, lacking the expression of receptors targeted successfully in other breast cancer subtypes. Treatment response to adjuvant and neoadjuvant chemotherapy is often short-lived and metastatic spread occurs at higher rates than other subtypes within the first five years after diagnosis. TNBCs exhibit stem cell features and are enriched for cancer stem cell (CSC) populations. E1A Binding Protein P300 (EP300) is a large protein with multiple cellular functions, including as an effector in stem cell biology.

**Methods:** We used a genetic knockdown (KD) model of EP300 in TNBC cell lines to investigate the effect on CSC phenotype, tumor growth and metastasis. Side population assay and tumorsphere suspension culture were used *in vitro*. Xenograft mouse models were used for *in vivo* studies. We performed *in silico* analysis of publicly available gene expression data sets to investigate CSC gene expression and molecular pathways as well as survival outcomes associated with EP300 expression in patients with TNBC and basal-like BC.

**Results:** EP300 KD abolished the CSC phenotype by reducing ABCG2 expression, side population cells and tumorsphere formation capacity *in vitro* as well as tumor formation in a xenograft mouse model *in vivo*. Metastatic capacity was markedly reduced in EP300 KD cells in vivo, with no detection of circulating tumor cells. TCGA data analysis demonstrated that genes positively correlated with EP300 expression in TNBC and basal-like BC were associated with CSC biology. Survival analysis demonstrated that EP300 expression predicts poor recurrence free survival in TNBC and basal BC.

**Conclusion:** We report a novel oncogenic role for EP300 in driving CSC phenotype representing a potential target to address tumor initiation and metastatic spread in TNBC and basal-like BC. EP300 might serve as a prognostic marker and potential therapeutic target in TNBC.

**Background**

Breast cancer (BC) is a heterogeneous disease with distinct subtypes each with different pathological and molecular features, variable responsiveness to therapy and clinical outcomes. Approximately 10–20% of all breast cancers are triple negative (TN) and lack the expression of estrogen and
progesterone receptors as well as the human epidermal growth factor receptor (HER) 2.

Early disease progression and metastasis often occurs within the first 5 years after diagnosis. The lack of targeted therapy options contributes to worse survival for TNBC, despite receipt of cytotoxic chemotherapy. [1–3]. Non-targeted neoadjuvant and adjuvant cytotoxic chemotherapy remains as the pharmacological backbone in the clinical management of TNBC. Targeted therapies for TNBC are an urgent unmet need in the breast cancer research community and for patients affected by the disease. More recently, the Food and Drug Administration (FDA) approved poly-ADP-ribose polymerase (PARP) inhibitors for a subset (10–15%) of TNBCs harboring germline mutations in BRCA1/2 [4] that work via synthetic lethality in this subgroup [5]. Immunotherapy using immune checkpoint inhibitors alone or in combination with chemotherapy and targeted therapies are approved [6] or under active investigation (e.g. TOPACIO/KEYNOTE-162, KEYNOTE-086, KEYNOTE-173).

Molecular studies led to a more detailed understanding of TNBC by defining distinct subtypes with clinical and therapeutic implications, showing that these cancers often exhibit basal-like features and are enriched for cancer stem cell (CSC) populations [7–9]. CSCs are thought to be responsible for tumor initiation, drug resistance and metastasis [10] and were first described in BC by Al-Hajj et al. [11]. CSCs can be identified by the expression of ATP-binding cassette transporters (e.g. MDR1 or ABCG2) as a side population in FACS assays [12].

EP300 (E1A-associated protein p300) is a large (~ 300KDa), multi-domain protein that functions as a histone/lysine acetyl transferase and chromatin remodeler as well as acetyltransferase for non-histone targets [13]. As part of various transcriptional complexes EP300 has important and diverse biological functions in cellular proliferation, cell cycle regulation, apoptosis, DNA damage repair, cell fate determination and stem cell pluripotency [14]. EP300 has been implicated in cancer biology [15], and has been described as both a tumor suppressor [16, 17] and more recently as an oncogene promoting tumor growth, metastatic potential and CSC phenotype in BC [18, 19].

We hypothesized that EP300 plays an oncogenic role in TNBC biology by regulating CSC populations. We show that knockdown of EP300 abrogates the CSC phenotype of TNBC cells and abolishes tumor growth and metastasis.
Methods

MDA-MB-231, Hs578T and BT20 cell lines were obtained from the American Type Culture Collection (ATCC) (Manassas, VA) and cultured in Dulbecco's Modified Eagle Medium (DMEM) (Thermo Fisher Scientific, Canoga Park, CA) + 10% fetal bovine serum (FBS) (Atlanta Biologicals, Flowery Branch, GA) + 1% Antibiotic-Antimycotic mix (Thermo Fisher Scientific). CAL51 was obtained from The Leibniz Institute DSMZ - German Collection of Microorganisms and Cell Cultures GmbH (Braunschweig, Germany) and cultured in DMEM + 20% FBS. Cell cultures were maintained and propagated in cell culture flasks (Corning Inc., Corning, NY) in a humidified incubator at 37 °C and 5% CO₂. To mimic in vivo conditions more closely, cells were also cultured in low glucose (1000 mg/L) DMEM (Thermo Fisher Scientific) and under low oxygen conditions (2%). For tumorsphere suspension culture, 20,000 cells per well were cultured in MammoCult culture medium (STEMCELL Technologies, Vancouver, Canada) in ultra-low adherence 6-well culture plates (Corning Inc.) for up to 14 days.

Stable and transient transfection

Lipofectamine 2000 reagent (Thermo Fisher Scientific) was used for stable and transient gene knockdown (KD) according to the manufacturer's protocol. Before transfection, cells were seeded at 3 × 10⁵ cells per well in 6-well plates or 1 × 10⁵ cells per well in 24-well plates and incubated with transfection medium for up to 48 h. For stable knockdown EP300 and scramble shRNA vectors were used (Thermo Fisher Scientific). For selection of cell clones with successful DNA integration, KD cells were further cultured as single cells in 96-well plates under the addition of G418 (geneticin) at 50 µg/ml. siRNA transient gene KD for EP300 and negative control were performed in duplicate using Silencer Select siRNAs (Thermo Fisher Scientific). As a control for transfection efficiency, the cells were simultaneously transfected with BLOCK-iT Alexa Fluor Red Fluorescent Oligo (Thermo Fisher Scientific) and red fluorescence expression was validated after 24 h transfection using a Zeiss Axiovert 200 inverted microscope. Gene knockdown was validated via qRT-PCR.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA extraction was performed using TRIzol reagent (Thermo Fisher Scientific) according to the manufacturer's protocol. First strand synthesis was performed using the qScript cDNA Supermix (Quantabio, Beverly, MA) and 1 µg of total RNA per reaction. The following reaction conditions were
used on a T100 thermal cycler (Bio-Rad, Irvine, CA): 25 °C for 5 min, 42 °C for 30 min, 85 °C for 5 min. Samples were prepared as duplicates unless stated otherwise.

For gene expression quantification, SYBR Green RT-PCR master mix was used (Quantabio). Reaction were performed in 96-well plates (Bio-Rad), using 2 µl cDNA, 12.5 µl SYBR Green master mix, 1µL each forward and reverse primer probes and 8.5 µl nuclease free deionized water. The following reaction conditions were used on a MyiQ or CFX96 Real-Time system (Bio-Rad): 95 °C for 3 min, 40 cycles of 95° C for 20 s, 60 °C for 20 s, 72 °C for 30 s. Primer sequences were obtained via the Harvard primer bank and synthesized by ValueGene (San Diego, CA). To ensure gene-specific priming, the primer sequences were validated using the NCBI BLAST tool and only exon-junction spanning primers used. Primer sequences and corresponding genes are listed in supplementary table S2.

**Fluorescence Activated Cell Sorting (FACS)**

Cells were harvested using non-enzymatic cell dissociation buffer (Sigma Aldrich, St. Louis, MO). FACS was performed on a BD LSRFortessa (BD Biosciences, San Jose, CA) using a 405 nm excitation wavelength. Gating was performed based on negative controls as described below. **Cell cycle analysis:** Cells were washed twice in ice-cold FACS buffer (PBS + 2% FBS) and centrifuged at 1000 rpm for 5 min between washes. After the second wash, the cell pellets were re-suspended in ice cold 80% ethanol. The ethanol was added dropwise under constant vortex agitation, to a final volume of 500 µl ethanol per 1 x 10⁶ cells. Samples were stored at 4 °C until further use. For analysis, 10 µl of a 1 µg/ml 4′,6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich) nuclear dye solution was added to a 100 µl ethanol cell suspension.

**Side population assay:** The cell number was adjusted to 1 x 10⁶ cells per mL and re-suspended in complete culture medium (DMEM + 10% FBS + 1% pen strep). Five µg of Hoechst 33342 dye (Sigma Aldrich) was added to each sample. For negative controls, samples were prepared in the same way, plus the addition of 100 µM verapamil (Sigma-Aldrich) to block dye efflux. The samples were incubated for 2 h at 37 °C in a cell culture incubator and gently agitated every 30 min. Following incubation, samples were immediately placed on ice and centrifuged at 1000 rpm at 4 °C for 5 min. Cell pellets were re-suspended in 300 µl cold PBS containing 5 µg/ml propidium iodide (PI) (Thermo
Fisher Scientific) for dead cell exclusion.

**Protein quantification**

Cells were washed twice with ice-cold PBS and subsequently scraped into ice-cold PBS plus protease inhibitor (Merck Millipore, Billerica, MA) and 1 µM Dithiothreitol (DTT) (Sigma-Aldrich), pelleted at 1000 rpm for 5 min and stored and – 80 °C until further use.

For protein extraction, the cell pellets were thawed on ice and processed using the Pierce M-PER Extraction Kit (Thermo Fisher Scientific) according to the manufacturer’s instructions. To ensure loading of equal amounts of protein for each sample, colorimetric protein quantification was performed using a Bio-Rad Protein Assay (Bio-Rad, Hercules, CA) and a SpectraMax M3 spectrophotometer (Molecular Devices, Sunnyvale, CA). For standard curve values, an albumin standard (Thermo Fisher Scientific) dilution serious was used. Protein extracts were mixed 1:1 with 2x Laemmli Sample Buffer (Bio-Rad) containing 5% of 2-mercaptoethanol (Sigma-Aldrich) and boiled for 5 min. The samples were loaded onto pre-cast 4-20% gradient gels (PAGEr Gold Precast gels, Lonza, Basel, Switzerland) including a Precision Plus Protein Dual Color Standard (Bio-Rad) for size determination. After gel electrophoresis (running buffer: 25 mM Tris HCl, 193 mM glycine, 0.1% sodium dodecyl sulfate (SDS), pH 8.3) the separated protein fractions were transferred onto nitrocellulose membranes (Bio-Rad) (transfer buffer: 80% Tris-Borate buffer [0.089 M Tris, 0.089 M Borate, pH 8.3], 20% methanol, 25 mM Tris HCl, 193 mM glycine) at 4 °C overnight. The membranes were then blocked in 5% skim milk for 60 min. Primary antibody incubation was performed in blocking solution over night at 4 °C. Primary antibodies: polyclonal rabbit anti-human p300 (N-15), monoclonal mouse anti-human ACTB (H-102) (both from Santa Cruz Biotechnologies). Secondary antibody incubation was performed for 60 min at room temperature. Secondary antibodies: goat anti-mouse IgG HRP conjugated; and goat anti-rabbit IgG HRP conjugated (both from Santa Cruz Biotechnologies). Subsequently the membranes were incubated with HRP substrate (GE Healthcare Life Sciences, Pittsburgh, PA) for 5 min. and chemiluminescence was recorded using a ChemiDoc MP gel imaging system (Bio-Rad). ImageJ software was used for quantification. Samples were prepared in technical duplicates.
Animal experiments

Eight to ten weeks old female NOD scid gamma (NSG) mice (NOD-scid IL2Rgamma\textsuperscript{null}, The Jackson Laboratory, Bar Harbor, Maine) were used for all xenograft experiments. The animals were housed at the USC Zilkha Neurogenetic Institute vivarium, according to IACUC requirements (protocol number 11204). For tumor cell injection, the mice were anesthetized with 2.5-4% isoflurane (Santa Cruz Biotechnology, San Diego, CA) under continuous infusion via a nose cone. At the experimental endpoint (tumor size $\sim 1500\text{mm}^3$ or mice showing significant signs of sickness due to cumulative tumor burden) mice were sacrificed using carbon dioxide in an enclosed chamber, followed by cervical dislocation. All animal procedures adhered to the ARRIVE guidelines [20].

**Tumor cell line subcutaneous (s.c.) and tail vein injection:** For subcutaneous xenograft experiments, cells were prepared at a concentration of $1 \times 10^6$ cells per 100 µl in a 1:1 mix of 75 µL cell culture medium (DMEM/ F12) and 75 µl Matrigel basement membrane (BD Biosciences, San Jose, CA), for a final volume of 150 µl slurry. A 100 µl bolus of cell slurry was injected subcutaneously into the left hind flank region of each mouse and the puncture site sealed using degradable tissue adhesive (3M Health Care, St. Paul, MN). Tumor growth was monitored weekly using a digital caliper (VWR, Radnor, PA). Tumor volume was calculated using the formula $\text{Volume} = (\text{Width} \times 2 \times \text{Length})/2$. To investigate whether micro-environmental or paracrine factors affect tumor growth of MDA-MB-231\textsuperscript{EP300KD} cells, wild type cells expression mCherry and MDA-MB-231\textsuperscript{EP300KD} clone 1 (GFP positive) were injected as a 1:1 mix for a total of $1 \times 10^6$ cells per mouse.

For tail vein injections, the mice were placed under a heating lamp for 2 min to dilate blood vessels. After subsequent immobilization in a rodent holder (Kent Scientific, Torrington, CT) $1 \times 10^6$ cells in 100 µl culture medium were injected per mouse using hypodermic syringes.

**In vivo imaging:** Tumor growth and spread after tail vein injection was monitored intra-vitally once per week for a total of 5 weeks. Luciferin (Promega, Madison, WI) was prepared at a final concentration of 300 mg/ml. After induction of anesthesia as described above, 300 µl of luciferin was injected intraperitoneally per mouse and allowed to distribute through the body of the animal for
15 min before imaging. Imaging was performed using an IVIS Spectrum pre-clinical in vivo imaging system (PerkinElmer, Waltham, MA) under continued anesthesia. Images were taken after 10 s, 1 min and 3 min each for supine and prone position. Bioluminescence was quantified using the Living Image 4.2 software (PerkinElmer).

Gene association and survival analysis
For gene association studies publicly available data sets from the The Cancer Genome Atlas (TCGA) breast cancer cohort were used and analyzed via Ingenuity pathway (IPA) tool and cBioPortal [21–23]. The Kaplan-Meier (KM) plotter web interface was used to assesses the effect of EP300 gene expression on survival in breast cancer patients [24]. KM plotter is a meta-analysis-based biomarker assessment tool using a manually curated gene expression database downloaded from Gene Expression Omnibus (GEO), European Genome-phenome Archive (EGA) and The Cancer Genome Atlas (TCGA). The auto selected cut-off was used to define high vs. low expression, which computes the best threshold between the upper and lower quartiles based on sample selection. Recurrence free survival (RFS) was reported at 60-month survival.

Statistical analysis
GraphPad PRISM (GraphPad Software Inc., La Jolla, CA) was used for statistical analysis of all experiments. Non-normal distribution was assumed, and appropriate non-parametric statistical tests were used; for experiments with single variables and groups of two Mann-Whitney test was used; for three or more groups, non-parametric one-way ANOVA (Kruskal-Wallis and Dunn’s multiple comparison) was used. For two variables and groups of two or more two-way ANOVA was used. Statistical hypothesis testing (Sidak test) was used to correct for multiple comparison. Data for repeats were presented as means and standard deviation. A two-tailed statistical significance level (alpha) of 0.05 for all statistical tests was considered meaningful to reject the null hypothesis. For survival analysis after tail vain injection, Kaplan-Meier curves were created and log-rank (Mantel-Cox) tests were used.

Results
Knockdown of EP300 in MDA-MB-231 cells leads to down-regulation of ABCG2 expression and elimination of the side population CSC phenotype

Reduction in protein levels after EP300 knockdown in MDA-MB-231\textsuperscript{EP300KD} clones compared to wild type (WT) and a non-specific scramble control (MDA-MB-231\textsuperscript{EP300KD} clone 1: 27% KD, clone 2: 63% KD) are shown in Fig. 1A (for full membrane image see additional file 1). Stable EP300 KD in MDA-MB-231 cells as well as transient KD in three other TNBC cell lines (BT20, CAL51 and Hs578T) reduced mRNA expression of ABCG2 in all but one cell line (Hs578T) (Fig. 1B and 1C). The Hoechst 33341 dye efflux assay (side population assay) demonstrated that both MDA-MB-231\textsuperscript{EP300KD} clones were depleted of cells with side population (SP) phenotype (scramble: 1.5%, MDA-MB-231\textsuperscript{EP300KD} clone 1: 0.1%, clone 2: 0%) (Fig. 1D). In summary, these results showed that EP300 KD led to a strong reduction in the expression of the CSC marker ABCG2 and abolished cells with CSC phenotype characterized by the side population in TNBC cells.

Tumorsphere formation capacity is reduced after EP300 KD in TNBC cells

MDA-MB-231\textsuperscript{EP300KD} clones showed significantly reduced tumorsphere formation capacity \textit{in vitro} (scramble 78 ± 5, KD clone 1 10 ± 2 n.s., KD clone 2 2 ± 3 \(p = 0.02\), \(n = 3\) each) (Fig. 2A). Transient siRNA-mediated KD of EP300 in two additional TNBC cell lines (BT20 and Hs578T) also showed reduction of tumorsphere formation, albeit to a lesser degree than stable KD (BT20 KD 43 ± 6 vs. scramble 77 ± 14, \(p = 0.018\); Hs578T KD 47 ± 6 vs. scramble 72 ± 5, \(p = 0.005\); \(n = 3\) each) (Fig. 2B). These results demonstrated that anchorage independent sphere formation as a characteristic of cell with CSC phenotype is reduced by EP300 knockdown to a varying degree in TNBC cell lines.

Knockdown of EP300 abolishes tumor formation and causes G2/M blockade in vivo

While mice bearing xenografts of MDA-MB-231\textsuperscript{WT} and scramble cells developed large tumors within three weeks of cell injection, tumors in MDA-MB-231\textsuperscript{EP300KD} cell xenograft bearing mice barely grew (tumor volume: WT 756.34 ± 58.34 mm\(^3\) scramble 978.18 ± 155.87 mm\(^3\), EP300 KD clone 1 31.54 ± 10.67 mm\(^3\), EP300 KD clone 2 68.17 ± 10.28 mm\(^3\)) (Fig. 3A and B). In contrast, MDA-MB-231\textsuperscript{WT} and EP300 KD cells showed similar growth dynamics in standard 2D monolayer culture \textit{in vitro} (Fig. 3C). MDA-MB-231\textsuperscript{EP300KD} cells from dissociated tumors after xenograft culture resumed regular growth dynamics in 2D culture (Fig. 3D). FACS based cell cycle analysis
demonstrated that both MDA-MB-231\textsuperscript{EP300KD} and scramble transfected cells showed similar cell cycle phase profiles under \textit{in vitro} conditions. MDA-MB-231\textsuperscript{Scramble} cells isolated after xenograft culture \textit{in vivo} showed a similar cell cycle profile to \textit{in vitro} cultured cells, while MDA-MB-231\textsuperscript{EP300KD} cells displayed a cycle profile indicative of a G2/M block after \textit{in vivo} culture (Supplementary fig. S1). Altered metabolic conditions more closely mimicking \textit{in vivo} settings (low glucose, 2\% low oxygen) demonstrated no statistically significant difference in growth behavior of MDA-MB-231\textsuperscript{EP300KD} compared to MDA-MB-231\textsuperscript{WT} or scramble cells \textit{in vitro} (Supplementary fig. S2). Similar tumor formation was observed in the mixed xenograft (MDA-MB-231\textsuperscript{EP300KD} plus MDA-MB-231\textsuperscript{WT}) and MDA-MB-231\textsuperscript{WT} cells only xenografts, without any detection of EP300 KD (GFP positive) cells in the mixed xenograft (tumor volume: WT \(1076.04 \pm 257.3 \text{ mm}^3\), mixed xenograft \(1178.82 \pm 405.16 \text{ mm}^3\), EP300 KD clone 1 \(13.5 \text{ mm}^3\))(Fig. 3E). Post xenograft FACS analysis of dissociated tumors revealed that only mCherry positive (WT) cells were present in the mixed xenograft, indicating that tumor growth was due to wild type cell growth only (Fig. 3F). Taken together, the data showed that EP300 KD inhibited tumor initiation/growth \textit{in vivo} but did not affect the growth behavior of KD cells \textit{in vitro} even under conditions partially mimicking \textit{in vivo} condition.

**EP300 KD reduces invasive phenotype and circulating tumor cells \textit{in vivo}**

After tail vein injection, MDA-MB-231\textsuperscript{EP300KD} cells expressing GFP/luciferase showed no tumor formation in the lungs or other parts of the mouse body \textit{in vivo} compared to large tumor masses (as determined by fluorescence signal intensity) in the control (scramble) group (RLU x \(10^5\): scramble \(2.6 \pm 3.19\), EP300 KD clone 1 \(0.006 \pm 0.004\), EP300 KD clone 2 \(0.004 \pm 0.003\) (Fig. 4A and B) (MDA-MB-231\textsuperscript{EP300KD} clone 1 vs. scramble \(p = 0.005\), clone 2 vs. scramble \(p > 0.0001\)). While all mice in the control group show significant signs of sickness, including low body weight and were sacrificed after 57.5 \(\pm 5.4\) days, mice injected with EP300 KD clones showed no sign of disease (Fig. 4C and supplementary fig. S3) (\(p = 0.0009\)). Post necropsy, blood samples of all mice were investigated for the presence of circulating tumor cells based on the presence of GFP-positive cancer cells in FACS analysis. While scrambled xenografted mice had 0.75 \(\pm 0.42\%\) GFP-positive cells in circulation, only 0.05 \(\pm 0.1\%\) and 0\% were found in MDA-MB-231\textsuperscript{p300KD} xenograft mice from clone 1 and 2, respectively (Fig. 4D) (\(p < 0.05\)). The results showed the EP300 KD abolishes metastatic colonization and spread in the form of circulating tumor cells in TNBC
cells.

**EP300 correlated genes show CSC related transcription in TNBC and basal-like BC**

The TCGA breast cancer cohort was filtered for TNBC (n = 82) or basal-like breast cancers (n = 81), which based on gene expression showed an overlap of n = 33 cases between both sample groups (Fig. 5A). Gene expression demonstrated that n = 3037 and n = 509 genes in TNBC and basal-like BC, respectively, correlated positively with EP300 expression (FDR < 0.05), with n = 298 genes correlating in both subgroups (Fig. 5B and supplementary table S1). The top associated pathways and biological functions in Ingenuity Pathway Analysis (IPA) (Qiagen, Hilden, Germany) are shown in Fig. 5B. While genes correlating with EP300 in basal breast cancer were associated with stem cell biology and cancer metastasis, EP300 correlated genes in TNBC were associated with growth factor signaling pathways (Fig. 5C). All three sets of genes positively correlated with EP300 (TNBC, basal-like BC and the overlap between both) were linked to PTEN and Protein kinase A (PKA) signaling (Fig. 5C).

Using the overlap gene signature (n = 298 genes) to analyze associated biological function via cBioPortal showed that the top associated pathways were TP53/BRCA, Notch and Wnt signaling (Fig. 5D and supplementary fig. S4). The expression of affected genes in these pathways (Notch – CREBBP, SPEN; Wnt – GSK3B) showed strong positive correlation with EP300 expression on a per sample basis (Fig. 5E). The graphs for Fig. 5D and E were created and directly exported from cBioPortal [23]. These results demonstrated that EP300 expression correlated with CSC related genes and pathways (Notch, Wnt, BRCA/TP53) suggesting at complex role of EP300 in CSC biology.

**EP300 is prognostic in TNBC and basal-like BC**

The association between EP300 expression and RFS was assessed in 6234 breast cancer patients, of which 198 TNBC cases were available for analysis. High EP300 gene expression was statistically significantly correlated with worse RFS in TNBC and basal-like BC patients with lymph node metastasis or high-grade tumors (G3) (Fig. 6).

Although not all results reached statistical significance, there was a clear trend towards increasing hazard ratios (HR) associated with higher histological grade (G3) and lymph node (LN) metastasis, with the highest HR in TNBC G3 and positive LN metastasis (Fig. 6). Comparing all PAM50 subtypes to basal-like BC had no significant effect on recurrence free survival. Of note, while higher EP300 RNA expression was associated with a better prognosis in BC in general (Supplementary fig. S5A), high EP300 protein expression demonstrated worse RFS (HR 3.32, 1.53-
7.19) in all BC patients (Supplementary fig. S5B). The results demonstrated that high EP300 RNA expression might function as prognostic marker for poor RFS in TNBC/basal-like breast cancer.

Discussion
Our findings shed new light on the role of EP300 in the cancer stem cell phenotype, tumor initiation and metastasis in TNBC. We demonstrated that knockdown of EP300 reduced the expression of ABCG2, eliminated side population cells and decreased tumorsphere formation potential in vitro. In vivo studies using stable EP300 knockdown cells showed a striking reduction in tumor initiation and tumor outgrowth. Knockdown cells also lacked the capacity for metastatic colonization potential and the formation of circulating tumor cell after tail vein injection. EP300 gene expression in large published breast cancer data sets positively corresponded with genes and molecular pathways associated with CSC function and predicted poor RFS in TNBC and basal-like BC. Collectively these data demonstrate a novel oncogenic role for EP300 in TNBC and basal-like breast cancer biology.

ATP Binding Cassette Subfamily G Member 2 (ABCG2), also known as the breast cancer resistance protein (BCRP) is involved in the trafficking of molecules across cellular membranes and has been demonstrated to play a pivotal role in drug resistance to chemotherapeutics used in the treatment of TNBC (e.g. anthracyclines and topoisomerase inhibitors) [25]. ABCG2 is a pivotal marker of side population cells, which have been shown to be enriched for cells with CSC characteristics in breast cancer [26, 27]. Our study showed a reduction in ABCG2 expression after EP300 knockdown and a strong abrogating effect on the side population phenotype in vitro as well as tumor initiation and lung colonization in vivo. Patrawala et al. showed that while the side population was enriched for tumorigenic CSC-like cells, ABCG2 levels did not affect their tumorigenic potential [26]. We showed that EP300 KD in Hs578T cells did not reduce the expression of ABCG2 but had a significant effect on tumorsphere formation. Other CSC markers have been proposed in breast cancer, such as the EPCAM⁺CD44⁺CD24⁻ phenotype [11], SOX2 [28], and ALDH1 [29]. It would be of interest to investigate the effect of EP300 KD on these cell populations as distinct CSC phenotypes seem to represent unique rather than common cell populations [30].

Further investigation into the underlying mechanisms by which EP300 knockdown inhibits tumor growth in vivo is warranted. As stromal cells play a pivotal role in tumor development [31], perhaps microenvironmental factors
such as paracrine signaling and physical cues from stromal cells affect tumor growth after EP300 knockdown [32]. Xenograft co-culture of MDA-MB-231\textsuperscript{EP300KD} with MDA-MB-231\textsuperscript{WT} did not stimulate outgrowth of KD cells \textit{in vivo}, yet these same knockdown cells exhibited regular growth behavior \textit{in vitro} before and after xenograft culture. Factors such as oxygen and glucose levels did not influence growth \textit{in vitro}. While stroma cells can function as tumor suppressors [33], it has been shown that factors from CSCs can corrupt these cells to promote cancer growth [34]. Conceivably after EP300 KD and reduced CSC-like phenotype, the balance of factors in the cancer cell microenvironment may be tipped towards the suppression of tumor outgrowth. The varying degree of reduction in tumorsphere formation capacity after EP300 KD also points towards a role of physical factors in the growth behavior of these cells. The observed differences in tumorsphere formation capacity after EP300 KD between different TNBC cell lines could be due to technical differences in gene knock down (stable vs. transient) or have distinct biological reasons such as the claudin-low versus basal-like subtypes [35].

Since we used an immunocompromised (NSG) mouse model, immunological factors are unlikely to play a role in the absence of \textit{in vivo} tumor formation through EP300 KD. Yet, as immunotherapies become increasingly important in the treatment of solid tumors, including BC [6], it will be of interest to investigate the effect of EP300 KD in TNBC cells in the context of a functional immune system [36]. For example, Xiong et al. showed that EP300 expression might be predictive of hyper progressive disease after PD-L1 blockade [37].

EP300 has been shown to increase migratory potential and invasiveness of cancer cells [38, 39]. Reduction in lung colonization and distant metastasis as well as circulating tumor cells after tail vein injection suggested that EP300 KD TNBC cells potentially either cannot survive in circulation or are unable to seed tumors at distant sites. It would be of translational interest to investigate whether EP300 targeted drugs (flufenamic acid [18], VV59 [19], A-485 [40]) can inhibit tumor initiation and metastasis in TNBC pre-clinical models.

TCGA analysis of TNBC and basal-like BC samples showed that genes and pathways associated with CSC biology were positively correlated with EP300 expression. Some examples are integrin and integrin-linked kinase (ILK) signaling [41], Wnt/\beta-catenin [42], actin-ARP-WASP [43], Notch signaling [44], BRCA [45] and embryonic stem cells (ES) transcriptional networks. The genes/signaling pathways common to TNBC, basal-like BC and the overlap between both were PTEN and protein kinase A (PKA). Both pathways have previously been described as regulators and potential targets in CSC biology [46, 47]. Other genes positively correlated with EP300 that
overlap between TNBC and basal BC (GSK3B, CREBBP, SPEN) and associated with the top affected pathways (Wnt and Notch signaling) have been linked to CSC biology and metastasis in breast cancer [48–50].

Survival analysis in an in-silico cohort of breast cancer cases demonstrated worse RFS correlated with high EP300 expression in TNBC and basal-like BC patients with high grade (G3) or LN metastasized tumors. Although not statistically significant due to small patient numbers, there was a trend toward increased hazard ratios for patients with both high grade and LN metastasized tumors. Validation of these results in larger patient cohorts as well as in BC patients with distant metastasis is warranted.

Several studies have shown that EP300 has tumor suppressor function [16, 17]. A specific mutation in EP300 (G211S) has been shown to be present in a subgroup of TNBC patients with very low overall mutational burden [51]. Interestingly, these patients had a lower risk of relapse and breast cancer-related mortality. Other groups demonstrated that EP300 may also function as an oncogene [18, 19]. Collectively, these findings raise the possibility that context matters. Another famous tumor suppressor, TP53, has been shown to potentially have pro-tumorigenic effects via increased inflammation [52] or anti-apoptotic mechanisms [53]. Similarly, we demonstrate here that EP300 might context-dependently favor a CSC phenotype.

Naturally there are limitations to our study. The fact that only a single cell line was used for in vivo studies, as well as the use of immunocompromised mice limits the extrapolation of our results to a more generalizable situation found in individual BC patients. The effect of the immune system on tumor growth is one of the most exciting and promising area of cancer research and the effect of EP300 expression in this context should be explored. The role of the tumor microenvironment more generally seems to play a critical role in the findings described here and invites further detailed experimentation.

Conclusion
Taken together, the data presented here is to our knowledge the first study describing an oncogenic role of EP300 in CSC phenotype in TNBC and basal-like breast cancer. We show that knockdown of EP300 abrogates the CSC phenotype of TNBC cells and abolishes tumor growth and metastasis. Our results warrant further exploration of EP300 as a prognostic factor and potential therapeutic target in TNBC and basal-like BC.

Abbreviations
ABCG2 – ATP-binding cassette super-family G member 2
Declarations

Ethics approval and consent to participate:
The animals were housed at the USC Zilkha Neurogenetic Institute vivarium, according to IACUC requirements (protocol number 11204). All procedures were approved by the Institutional Review Board (IRB) at the University of Southern California (USC).

Consent for publication:
not applicable

Availability of data and materials:
The gene expression datasets analyzed during the current study are publicly available in the cBioPortal and KM plotter as referenced in the manuscript. All data generated or analyzed during this study are included in this published article (and its Supplementary Information files).

Competing interests:
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**Figures**
EP300 KD reduces ABCG2 levels and side population cells. A, Western plot protein quantification of EP300 and ACTB in MDA-MB-231WT, non-specific scramble shRNA and two stable shRNA EP300 KD clones (n=1 each). B, ABCG2 gene expression in MDA-MB-231 cells after scramble (n=1) or EP300 specific stable transfection (n=2). C, ABCG2 gene expression after transient siRNA KD of EP300 in three TNBC cell lines (BT20, CAL51, Hs578T) (n=2 per cell line). D, FACS side population (SP) quantification in the MDA-MB-231EP300KD (n=2) and scramble (n=1).
Figure 2

EP300 KD reduces tumorspheres formation. A, Tumorsphere assay (14 days in culture) of MDA-MB-231EP300KD and scramble clones (n=3 per condition). B, Tumorsphere assay (7 days in culture) after transient EP300 KD (48h) in two TNBC cell lines (BT20 and Hs578T) (n=3 per cell line per condition).

(*p<0.05, **p<0.01).
Figure 3

MDA-MB-231EP300KD does not affect proliferation in vitro but abolishes tumor growth in vivo. A, Growth dynamic of cells in NSG mice (MDA-MB-231WT, MDA-MB-231EP300KD clone 1 and 2, n=5 per cell line),
p-values (1) - WT vs. EP300 KD clones, (2) - scramble vs. EP300 KD clones. B, Representative images of explanted tumors post xenograft culture. C, Growth dynamics (4 days) in vitro of MDA-MB-231WT (dotted line) and MDA-MB-231EP300KD (n=3 per cell line). (***p<0.001, ****p<0.0001). D, Growth curve in vitro of MDA-MB-231EP300KD (dotted line) post xenograft (n=3 per cell line). E, Growth dynamic of MDA-MB-231 xenograft in female NSG mice (MDA-MB-231WT, EP300 KD clone 1, mixed xenograft (WT and EP300 KD clone 1 mixed 1:1), n=5 mice per cell line. F, FACS analysis of cell composition after mixed xenograft experiment (WT - mCherry, EP300 KD – GFP).
Figure 4

EP300 KD reduces cell metastatic colonization and circulating tumor cells in vivo. A, In vivo bioluminescence images of mice bearing xenografts of MDA-MB-231Scramble and EP300 KD (clone 1 and 2) 5 weeks after tail vein injection (n=4 for scramble, n=5 for EP300 KD clones each). B, Quantification of bioluminescence measurements (post 5 weeks). C, Survival curve for mice injected via the tail vein with MDA-MB-231Scramble or EP300 KD. D, Circulating tumor cell quantification based on GFP expression via FACS in peripheral blood of mice post tail vein injection with MDA-MB-231Scramble or EP300 KD cells (same mice as shown in Figure 4D). (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).
Figure 5

EP300 expression is correlated with CSC gene expression in the TCGA BC cohort. A, Overlap between TNBC (n=82) and basal-like BC (n=81). B, Number of genes positively correlated with EP300 in basal-like BC (n=509) and TNBC (n=3036) and overlap of genes (n=298) (q-value < 0.05). C, Biological functions and pathway associated with the significantly correlated genes (IPA analysis), ranked by p-value (range 1.74x10-4 to 1.34x10-3). Grey arrows indicate overlapping functions between gene lists. D, Affected biological functions and pathways (BRCA/TP53, Notch) associated with EP300 correlated genes that overlap in TNBC and basal BC (n=298) (cBioPortal). Affected genes are outlined in bold.
black. Percentages represent the proportion of samples which show alterations in pathway genes (bold - TNBC cases, regular font - basal-like BC). E, Bivariate plots of genes in affected pathways (D) that are positively correlated with EP300 expression (RSEM – RNA-Seq by Expectation-Maximization).

Figure 6

EP300 expression predicts recurrence free survival: Kaplan-Meier analysis showing RFS in EP300 high vs. low expressing TNBC and basal BC patients in association with tumor grade (G3 – high grade vs. all grades), LN metastasis (LN all vs. LN metastasis) and PAM50 subtypes (all vs. basal-like TNBC) (bold black framed Kaplan-Meier plots with p<0.05).

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