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Ly6E/K signaling to TGF-β promotes breast cancer progression, immune escape and drug resistance

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Abstract

Stem cell antigen Sca-1 is implicated in murine cancer stem cell biology and breast cancer models, but the role of its human homologues Ly6K and Ly6E in breast cancer are not established. Here we report increased expression of Ly6K/E in human breast cancer specimens correlates with poor overall survival, with an additional specific role for Ly6E in poor therapeutic outcomes. Increased expression of Ly6K/E also correlated with increased expression of the immune checkpoint molecules PDL1 and CTLA4, increased tumor-infiltrating T regulatory cells and decreased natural killer (NK) cell activation. Mechanistically, Ly6K/E were required for TGF-β signaling and proliferation in breast cancer cells where they contributed to phosphorylation of Smad1/5 and Smad2/3. Further, Ly6K/E promoted cytokine-induced PDL1 expression and activation and binding of NK cells to cancer cells. Lastly, we found that Ly6K/E promoted drug resistance and facilitate immune escape in this setting. Overall, our results establish a pivotal role for a Ly6K/E signaling axis involving TGF-β in breast cancer pathophysiology and drug response, and highlight this signaling axis as a compelling realm for therapeutic invention.

Introduction

In recent years, it has become increasingly clear that the process of breast cancer oncogenesis and therapeutic sensitivity is profoundly affected by pathways that also infringe on cancer stem cell biology(1,2). One of the gene families at the interface of cancer stem cell biology and cancer biology is the human Ly-6 gene family, which is related to the murine

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stem cell antigen-1 gene (Sca-1) or mouse (m) Ly6A. It encodes a set of glycosylated cell surface proteins that have been recognized as stem cell markers (1,3,4). The mLy6A confers resistance to radiotherapy and promotes metastatic behavior of mammary tumors in animal models (5). Earlier work from our laboratory has demonstrated that mLy6A regulates TGF-β, PTEN, and ERK/AKT cell signaling pathways (6–8). Mechanistically, mLy6A binds to TGF-β receptor-1 (TβR1), disrupts the TβR1 ligand complex and inhibits Smad2/3 signaling. In addition, increased mLy6A expression in tumor cells correlates with a reduced level of GDF10, a novel tumor suppressive cytokine (6). Recently, Ly6K and Ly6E – another members of the Ly6 gene family – have been shown to be involved in human malignancies and have been suggested as potential therapeutic targets for cancer immunotherapy (9–14). Here we set out to investigate their role in breast cancer progression and the underlying cellular signaling mechanisms and relate these findings to clinical cases of breast cancer using clinical informatics.

Materials and Methods

Bioinformatics analysis

Oncomine (www.oncomine.org) was used to visualize gene expression microarray dataset. ProgeneV2 online tool (http://www.abren.net/PrognoScan/) was used to study survival outcome.

TCGA Breast Dataset: http://tcga-data.nci.nih.gov/tcga/.

Reagents and Reporter Plasmids

TGF-β1, IFN-γ and IL4 was obtained from R&D Systems. The Smad-responsive TGF-β reporter plasmids is as described before(6).

shRNAs and Cell Lines

Ly6K sh1 (Cat# TRCN0000117952), Ly6K sh2 (Cat# TRCN0000117953), Ly6E sh1 (Cat# TRCN0000154460), and Ly6E sh2 (Cat# TRCN0000155331) shRNAs cloned into pLKO.1 were obtained from Sigma Inc. Lentivirus was produced in 293T cells by cotransfection of the pMD2.g and VSVG vectors. At 24 h after transfection, the medium was replaced and virus was collected. Cells were infected with lentivirus for 24 h in the presence of 4 mg/mL of polybrene, and selection carried out with 1 μg/mL of puromycin.

For overexpression, the open reading frame containing clones (Ly6K, Ly6E) in G418 selection markers were obtained by Origene MD. The cells were transfected using Fugene HD and selected in 1mg/ml G41.

The KHYG-1 natural killer cell line stably expressing KIR2DS1*002 have been generated and used as described previously (37). Prior to each assay cells are screened for the surface expression of KIR2DS1 by immuno-labeling using 2DS1 MoAB--CD158a/h (clone EB6B or 11PB6) and flow assay. Zeocin is removed prior to NK cell coculture assays.

The cells were obtained by American Type Cell Culture, which provided cells authenticated by short random repeat DNA sequencing. Upon arrival cells were propagated and stored in

multiple vials as recommended. Each vial was cells were used and discarded within six months.

Colony assay

Cells (5,000/dish) were seeded into 100-mm dishes in 10 mL of DMEM containing 5% FBS. After 7–10 d, colonies were fixed in 50% trichloroacetic acid, stained with sulforhodamine B, and solubilized in 10 mM Tris-HCl (pH 10), and absorbance was measured at 560 nm(6).

Nude mice xenograft assay

Five-week-old athymic nude mice were injected sub cutaneous (s.c.) into opposite flanks with 500,000 cells. For the mice receiving T47D cells, mice were transplanted with controlled-release (60 days release time, 0.18mg/pellet total dose) 17β-estradiol pellet (Innovative Research of America, Sonnasota, FL). Tumor volume was determined by caliper measurements at weekly intervals. All animal studies were performed under a protocol approved by Georgetown University's Animal Care and Use Committee.

Quantitative Real-Time (qRT) PCR

Total RNA was extracted using the Qiagen RNAeasy Mini Kit and cDNA was prepared according to the manufacturer's protocol (Invitrogen). qRT PCR was performed in triplicate in an ABI 7900 instrument using SYBRGreen detection (Applied Biosystems) according to the manufacturer's protocol. All primer sequences are described (Table S1). The expression of each target gene was normalized to the expression of GAPDH using SDS2.4 software (Applied Biosystems)

Reporter assays

Cells were seeded in triplicate into 24-well plates at a density of 4×10^4 cells/well and then transfected with 100 ng of Smad responsive firefly luciferase reporter plasmid and 1 ng of pCMV Renilla luciferase (for normalization) using Fugene HD (Roche). Luciferase activity was measured at 48 h after transfection using the Dual Luciferase Reporter Assay System (Promega) and a Leader 50 Luminometer (Gen-Probe).

TGF-β, IFN-γ and IL4 treatment

Cells were seeded at 60% cell density and serum starved in 0.5% charcoal stripped serum over night. Cells were treated with 100pg/ml TGF-β1 for 30 min, 10ng/ml IFN-γ or 50ng/ml IL4 for overnight.

Western blot analysis

Cells were lysed in a buffer containing 60 mM Octylglucoside, 0.5% Nonidet P-40, 0.1% SDS, 0.25% sodium deoxycholate, 50 mM Tris-HCl (pH 7.5), 125 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10% glycerol, phosphatase and a protease inhibitor mixture (Roche Molecular Biochemicals) at 4 °C. 20–50 μg of lysate was separated in a 4–12% NuPAGE Bis-Tris gel (Invitrogen) and transferred to nitrocellulose membranes. Primary antibody was incubated for either 1.5 h at room temperature or overnight at 4 °C. Secondary antibody was incubated

for 30 min at room temperature, and proteins were visualized with West Pico Stable (Pierce). For Western blot analysis, the primary antibodies anti-Ly6K (cat # AF6648, R&D systems), Ly6E (cat # NBP1 68553, Novus Biologicals), pS423/425 Smad3 (cat # 9520), pS463/465 Smad1/5 (cat# 9516), Smad2/3 (cat #9523), Smad5 (cat #12534) were purchased from Cell Signaling.

Semi-quantitative immunohistochemistry (IHC)

IHC was performed using Tissue staining HRP-DAB system kits (R&D system). Antigen retrieval was performed using citrate buffer (10mM Citric acid, 0.05% Tween 20, pH6.0). The primary antibodies were used for 1 hour at room temperature using anti Ly6K antibody (AF6648, R&D systems) at 1:100 and anti Ly6E antibody (NBP1 68553, Novus Biologicals) at 1:400 dilution on clinical samples of breast cancer (Tissue microarrays, USBiomax, MD, Histopathology and Tissue Shared Resources at Georgetown University). Slides were semi quantitatively scored in a blind fashion using both intensity and percent positive cells. Intensity was scored on a scale of 0 to 3 as follows: 0= negative, 1= weak, 2= moderate, 3= intense. Percent positive labeling was scored on a scale of 0 to 3 as follows: 0-negative, 1= <10%, 2= 11–50% and 3= >50%. Both these values were added for final numerical score. A score of 2 and below was considered negative and a score of 3 and above was considered positive.

NK cell activation assay

NK cell activation was measured by the release of Granzyme B and the pro-inflammatory cytokine MIP-1α from the KIR2DS1*002 NK (KHYG-1) cell line (37,49). Briefly, 2DS1 NK cells were co-cultured with indicated cells at a ratio of 1:2 for 48 hours at 37°C in a 5% CO_2 environment. Each assay condition consisted of 1×10^5 effectors (2DS1 NK cells) and 0.5×10^5 targets in 200 μl culture media in one well of a 48 well plate. After 48 hours, the cell culture supernatant was collected and stored at −80C. Using the Luminex 100 platform (Luminex), secreted levels of granzyme B and MIP-1α were assayed via the Procarta Immunoassay Kit (eBiosciences, Affymetrix) according to the manufacturer's instructions.

Flow cytometry

Single-cell suspension primary cultures or cell lines were prepared by incubation with 0.05% trypsin for 1–2 min, filtering through a 40-μm cell strainer, two washes with 1× PBS, and blocking for 15 min on ice with 1× PBS supplemented with 3% FBS. Cells were washed twice with 1× PBS, fixed with 1% paraformaldehyde in PBS, and stored in the dark at 4°C until being sorted on a FACSAria flow cytometer using FACS Express De Novo software (BD Biosciences).

PDL1 surface expression assay

To assay the surface expression of PDL1, cells at a density of 5×10^6 cells/mL were incubated for 1 h at room temperature with 1:200 dilutions of a BV421 anti-human CD274 (PDL1) antibody (Biolegend) or IgG (of the same isotype was used as a negative control) to perform flow cytometry.

NK cell conjugate assay

2DS1 NK cells were labeled with Vybrant DiO Cell-Labeling Solution and the MDA-MB-231 control, Ly6K, and Ly6E knockdown cells were labeled with Vybrant DiI Cell-Labeling Solution according to the manufacturer's protocol (Life Technologies) and co-cultured for 48 hours. The cells were analyzed by flow cytometry. We calculated the % conjugate by obtaining the ratio of MDA-MB-231 bound to NK cells vs total live MDA-MB-231.

Statistics

Statistical significance ($P<0.05$) was determined from the mean±SEM using two-tailed Student t tests and between groups of two or more variables using two-tailed Fisher exact tests.

Results

Increased expression of Ly6K and Ly6E in breast cancer

Analysis of the normal tissue samples showed that Ly6K was highly expressed in Testis and Ly6E in liver tissue among the array of normal tissue samples (Figure S1A). Analysis of DNA copy number in invasive ductal breast carcinoma in TCGA dataset showed a significant genomic amplification of Ly6K and Ly6E in cancer tissue (Figure S1B). Statistical analysis across seven comparisons (cancer vs normal) in three independent studies TCGA (unpublished), Richardson (16) and Curtis(17) showed increased Ly6K and Ly6E in breast cancer (Figure S1C, Table S2).

Increased expression of Ly6K and Ly6E and overall patient survival

The status of gene expression and survival outcome was assessed by using PROGgeneV2 online tool which showed that increased Ly6K (Figure 1A) and increased Ly6E status (Figure 1B) correlate well with poor 5-year overall survival in a breast cancer study (18) and in the Netherland Cancer Institute (6,9–14,19–23) respectively.

We used comprehensive NKI dataset to study metastasis free survival. The NKI study did not detect Ly6K expression data, but provided us with a wide range of information regarding the status of Ly6E expression and therapeutic outcome. Increased Ly6E expression is correlated with a poor 5-year metastasis-free chemotherapy outcome (Figure 1C–D) and hormone therapy (Figure 1E–F).

Ly6K and Ly6E expression in a breast cancer cell lines and breast cancer tissue

We next screened a panel of breast cancer cell lines for the RNA expression and found that triple negative breast cancer (TNBC) cell lines expressed a higher levels of Ly6K (Figure 2A) while Ly6E expression was detected in all breast cancer cell lines with higher levels in ER positive breast cancer cell lines (Figure 2B). Ly6K and Ly6E protein expression was tested in breast cancer clinical specimens. An IHC study containing 52 clinical samples (n=29 ER+, n=23 TNBC) revealed that 87% of TNBC tumors express detectable levels of Ly6K while 24% TNBC tumors express Ly6E. 76% of ER positive tumors express Ly6E and 33% of ER positive tumors express Ly6K (Figure 2C).

Effect of Ly6K and Ly6E on breast cancer growth

To investigate the functional role of Ly6K in breast cancer cells, we chose to stably knockdown Ly6K in MDA-MB-231 TNBC cells (Figure 3A, Figure S2A). Ly6K knockdown cells gave rise to fewer colonies (Figure 3B, Figure S3A). Ly6K knockdown cells gave rise to fewer and smaller xenografts (Figure 3C). MDA-MB-231 cells exhibit a strong metastatic phenotype (24,25), so we examined the effect of Ly6K knockdown in these cells on the invasion and distant colonization of tumor cells in well characterized zebrafish model (26–28). The Ly6K knockdown cells showed diminished cell migration and colonization (Figure S4). To investigate the functional role of Ly6E in breast cancer cells, we chose to stably knockdown Ly6E in T47D ER positive cells (Figure 3D, Figure S2B). In colony assays, Ly6E knockdown cells gave rise to fewer colonies (Figure 3E, Figure S3B). T47D cells have been demonstrated to establish xenograft tumors when supplemented with estrogen (29). Ly6E knockdown led to reduced xenograft tumors (Figure 3F). These results suggest that Ly6K or Ly6E are required for tumorigenic growth.

Ly6K and Ly6E promote gene signatures associated with drug resistance and epithelial-to-mesenchyme transition (EMT) in breast cancer

We next examined whether Ly6K and Ly6E contribute to tumor growth by inducing characteristic gene signatures that lead to drug resistance or EMT. Ly6K knockdown in MDA-MB-231 and Ly6E knockdown in T47D cells significantly reduced RNA levels of the ABCC3 (Figure 4A), an inducer of chemotherapeutic drug-resistance (30), ABCG2 (Figure 4B) and FGF-7 (Figure 4C), inducers of hormonal therapy resistance in breast cancer (31,32). Moreover, Ly6K and Ly6E knockdown also reduced the RNA expression of NANOG (Figure 4D), CD34 (Figure 4E) and PSCA (Figure 4F), well-known stem cell genes that have been recently described to induce drug resistance (33–35). Next we tested for the effect of Ly6K and Ly6E on regulators of EMT pathway, which is closely related to drug resistance and tumor metastasis. MDA-MB-231 cells express higher levels of Zeb1, an EMT inducer than T47D cells (Figure 4G, compare first bar in main and inset graphs). The knockdown of Ly6K or Ly6E reduced expression of ZEB1 ((Figure 4G–J). Knockdown of either Ly6K or Ly6E decreased E-Cadherin and increased N-cadherin (Figure 4K), reversing the hallmark of EMT switch.

Ly6K and Ly6E are required for TGF-β signaling in cancer cells

We have previously shown that the mouse Ly6A/E (Sca-1) gene – a potential functional homologue of human Ly6E and Ly6K – regulate TGF-β/Smad2–3 signaling (6). We found that Ly6K and Ly6E are required for TGF-β/Smad signaling as seen by Smad2/3 specific reporter activity (Figure 5A). Depletion of Ly6K and Ly6E reduced the endogenous phosphorylation status of Smad3 (TGF-β signaling) and Smad1/5 (BMP signaling) suggesting that Ly6K and Ly6E are required for the increased TGF-β/Smad signaling in cancer cells (Figure 5B). Loss of Ly6K and Ly6E also reduced levels of the TGF-β responsive genes PAI1 (Figure 5C) and CTGF (Figure 5D). Next we investigated whether Ly6K and Ly6E are required for ligand-induced TGF-β/Smad signaling in cancer cells. Knockdown of Ly6K or Ly6E reduced phosphorylation levels of Smad1/5 and Smad3

(Figure 5E). These findings suggest that Ly6K and Ly6E are required for endogenous and ligand-induced TGF-β and BMP signaling in cancer cells.

Ly6K and Ly6E facilitate tumor cell escape from immune surveillance

TGF-β signaling plays an important role in the tumor microenvironment including the innate immune response generated by natural killer (NK) cells and the adoptive immune response carried out by cytotoxic T lymphocytes (CTL) (36). A statistical analysis of gene expression in across 6-comparisons (TNBC vs non TNBC, Normal vs Cancer) in three independent data sets showed Ly6K and Ly6E correlate significantly with breast cancer subset with increased PDL1, CTLA4 (tumor immune checkpoint inhibitors) and increased infiltration of suppressive T-regulator cells (marker CD25+) in Curtis(17), Gluck(50) and TCGA (Figure 6A, Figure S5).

To evaluate the effect of Ly6K and Ly6E knockdown on natural killer cell activation, we used NK cells expressing KIR2DS1 receptor (37) and MDA-MB-231 cells, which have HLA-C*02:02, C*17:01 (unpublished data from Drs. Hurley and Upadhyay, Georgetown University), the classical ligand for the stimulatory KIR2DS1 receptor found on a subset of natural killer cells. We observed that 2DS1 NK cells released significantly higher levels of Granzyme B and MIP1α when co-cultured with Ly6K and Ly6E knockdown cells (Figure 6B I, II), suggesting that high expression levels of Ly6K and Ly6E in cancer cells can reduce NK cell activation. Binding of NK cells to cancer cells precedes the NK cell activation and subsequent killer cytokine release like Granzyme B. We observed that knockdown of either Ly6K or Ly6E led to significantly increased binding of cancer cells to NK cells (Figure 6B–III, Figure S6).

Several cytokines including IFN-γ and IL4 may induced PDL1 expression on the cell surface of cancer cells is a major mechanism of tumor cell immune escape (38,39). We found that knockdown of Ly6K or Ly6E led to reduced stimulation PDL1 expression followed by IFN-γ and IL4 treatment (Figure 6C I–II, Figure S7), suggesting that Ly6K or Ly6E is required for cytokine-induced PDL1 expression by cancer cells. We found that rescue by forced expression of Ly6K or Ly6E increased colony growth, reduced Granzyme B release and increased PDL1 expression (Figure S8).

Discussion

Stem cell antigen-1, also known as mouse Ly6E/Ly6A, is an established marker of cancer stem cells (40–42). Sca-1 can regulate tumor suppressor cytokine GDF10 production by cancer cells and disrupt TGF-β signaling by directly sequestering TGF-β receptor-1(6). In spite of these recent findings, the role of *homo sapiens* Ly6 genes in the biology of human cancer progression remains poorly understood. In the present study, we have revealed several novel aspects of the human Ly6 genes Ly6K and Ly6E in the pathobiology of breast cancer progression and tumor immune escape. Our study reveal the following major findings: first, both Ly6K and Ly6E are show elevated expression levels at the RNA and protein level in human breast cancer; second, Ly6K or Ly6E are prognosis markers for poor overall patient survival; third, Ly6K or Ly6E are required for the growth of human breast cancer cells; fourth, Ly6K and Ly6E are required for endogenous and ligand-induced phosphorylation of

Smad2/3 and Smad1/5 in cancer cells; and fifth, Ly6K and Ly6E are required for tumor immune escape. Interestingly, knockdown of Ly6K or Ly6E was sufficient to induce phenotypic changes.

We found that high Ly6K and Ly6E expression and increased growth of cancer cells are associated with their ability to maintain TGF-β and BMP signaling in cancer cells. TGF-β signaling plays a central role in growth, metastasis, EMT and invasiveness of cancer cells (43–45) (46). The functional role of Ly6 family members, however, may be more complex than a linear regulation of a downstream pathway as highlighted by two findings: First, Ly6K and Ly6E expression is segregated in ER positive and triple negative breast cancer. Secondly, Ly6K and Ly6E are required for high expression levels of several markers of chemotherapeutic and hormonal drug resistance. The role of Ly6E in drug resistance was further supported by clinical survival data, that the expression of Ly6E may also be a marker of therapeutic response. Mechanistically, the differential expression of Ly6K and Ly6E and their effect in tumorigenic processes may be a function of an altered genetic network specific to certain tumor types, which can converge on TGF-β signaling. TGF-β signaling regulates both chemotherapy and hormonal therapy drug resistance (47,48).

The underlying mechanism of the noted phenotypic effects of Ly6K and Ly6E on tumor immune escape relevant to NK cell binding and, cytokine release and PDL1 expression involves critical essential roles of Ly6K and Ly6E for supporting constitutive TGF-β signaling and cancer cells cross-talk with tumor microenvironment especially in cancer immune surveillance (Figure 6D). These finding suggest that the Ly6 family is one of the nodal centers involved in the regulation of TGF-β signaling. Our findings suggest that Ly6K and Ly6E are likely to emerge as attractive upstream targets to specifically modify TGF-β signaling in cancer cells. We hope this approach will leave the TGF-β signaling intact in normal cells and simultaneously decrease the intrinsic ability of cancer cells to grow and increase the effectiveness of immune surveillance leading to an improved chemotherapy or hormonal therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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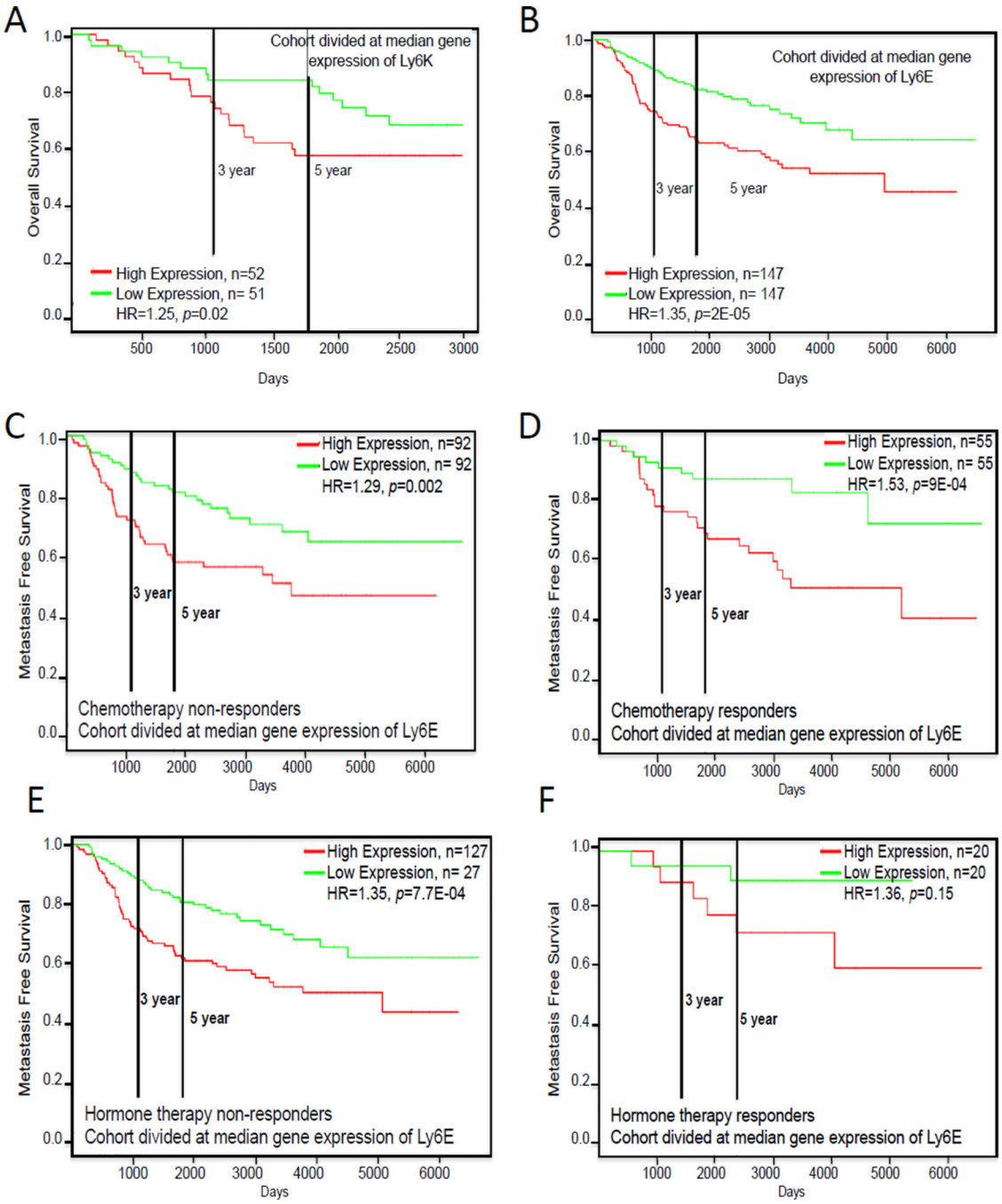

Figure 1. Ly6K and Ly6E are markers for poor prognosis in human breast cancer

(A) Increased Ly6K and **(B)** increased Ly6E expression in breast cancer samples is significantly associated with poor overall survival (end point - death) in the clinical cases of breast cancer. Increased Ly6E expression in breast cancer samples is significantly associated with poor metastasis free survival in **(C)** Chemotherapy non-responding group (n=104) and **(D)** chemotherapy responding group (n=110). Increased Ly6E expression is significantly associated with poor metastasis free survival in **(E)** hormonal therapy non-responders

(n=254) but not in **(F)** hormonal therapy responders (n=40). All graphs were generated using the PROGgeneV2 online tool. n = number of samples, HR = Hazard Ratio.

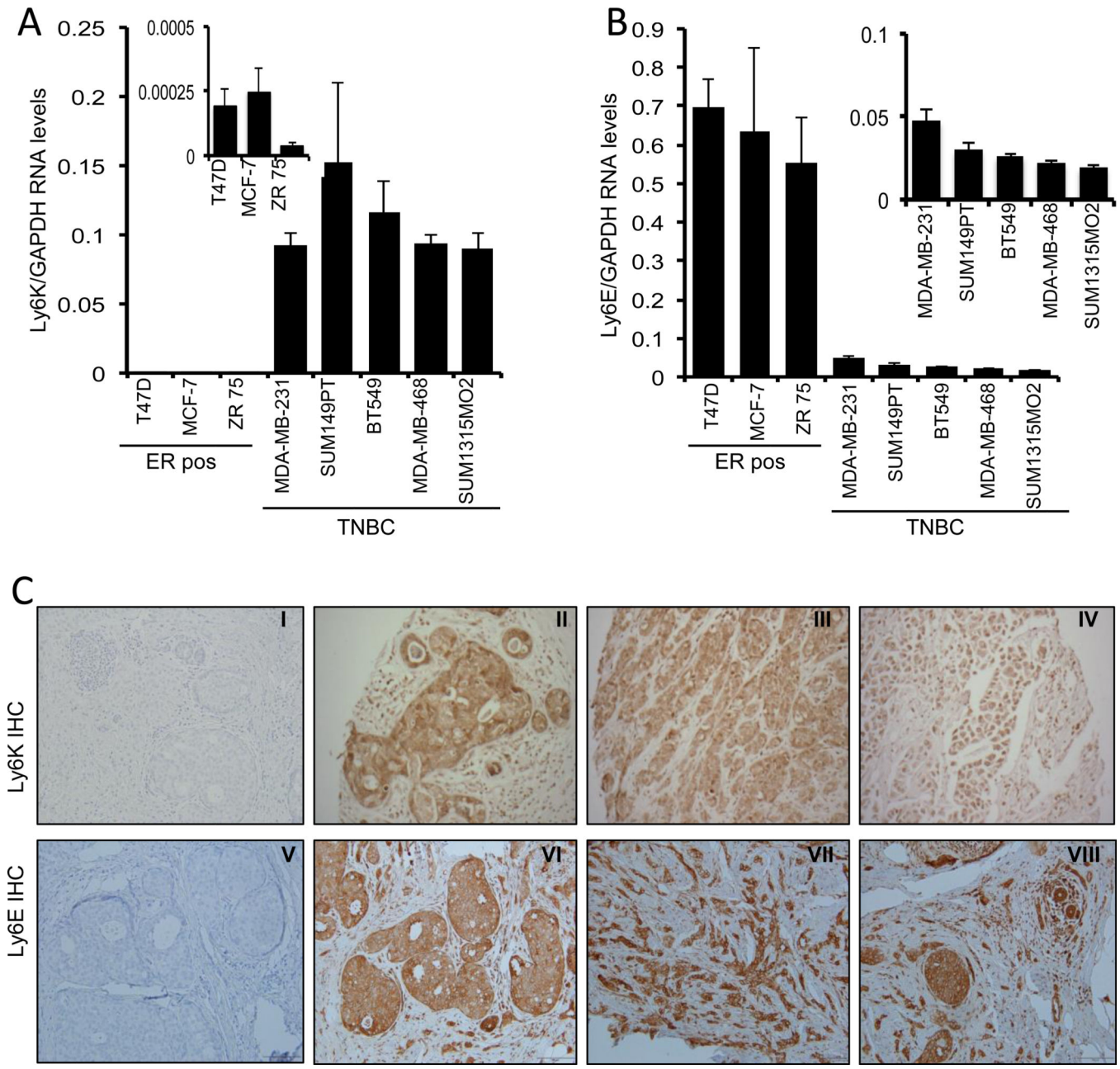

Figure 2. Ly6K and Ly6E are differentially expressed in breast cancer cell lines and cancer tissues

(A) qRT-PCR analysis shows that Ly6K is highly expressed in triple negative breast cancer (TNBC) cell lines. **(B)** qRT-PCR analysis shows that Ly6E expression is expressed in all breast cancer cell lines with a 7-fold higher expression in ER positive cell lines than in TNBC cell lines. **(C)** Example of a positive immuno-labeling of Ly6K (**II**-ductal carcinoma insitu (DCIS) ER+**, III**-invasive ductal ER+, **IV** -TNBC) and a negative control **(I)**; example of a positive immuno-labeling of Ly6E protein (**VI**-DCIS, ER+, **VII**-invasive/lobular TNBC, **VIII**-invasive and DCIS, ER+) and negative control **(V)**. Quantification chart (bottom panel)

shows % of high numerical grading of Ly6K and Ly6E in tested ER positive and TNBC cases.

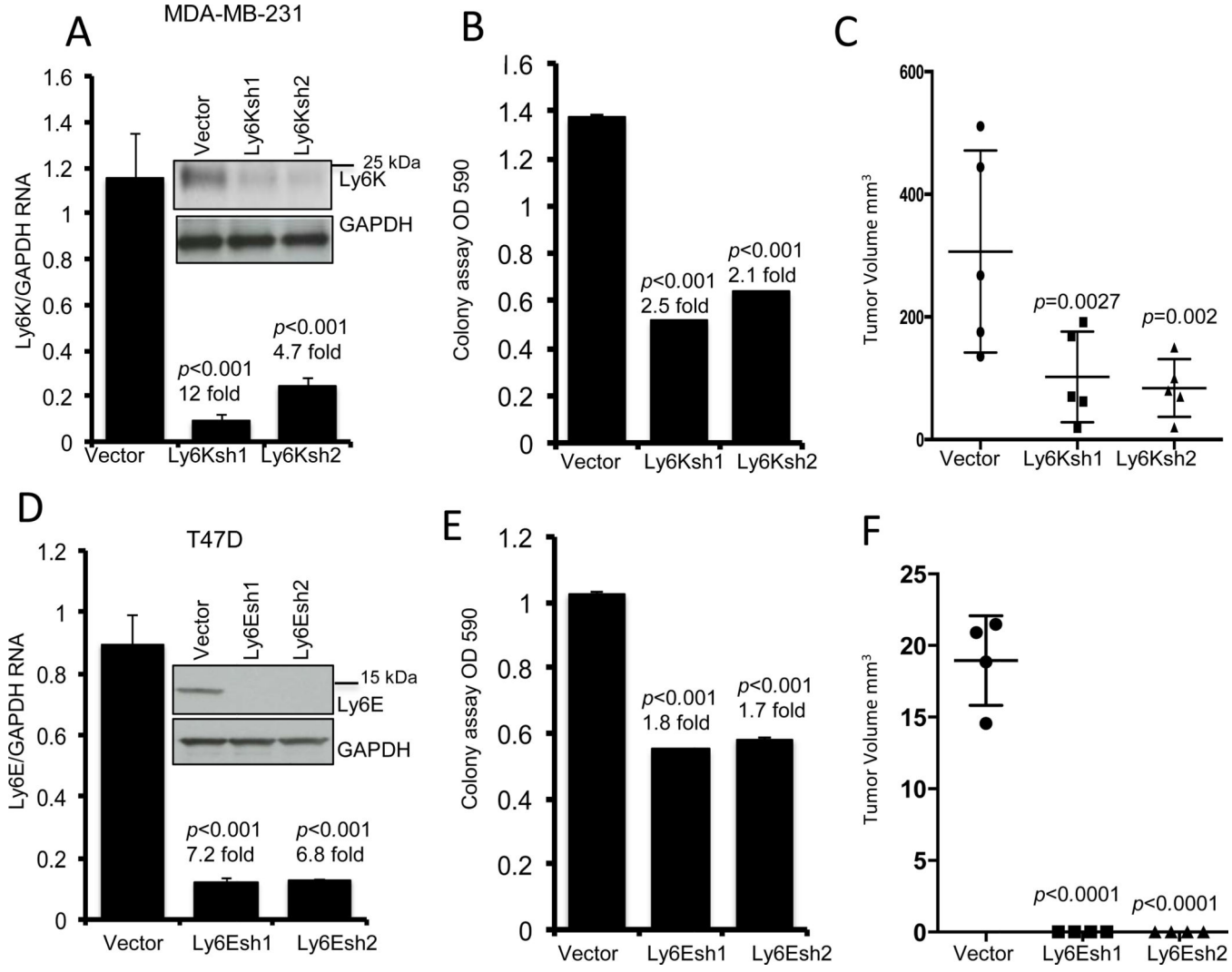

Figure 3. Ly6K and Ly6E are required for tumor cell growth

(A) The qRT-PCR and western blot shows Ly6K expression in the indicated cells. **(B)** Indicated cells were seeded in low dilution for colony assay. Ly6K knockdown cells have significantly reduced colony formation. **(C)** Indicated cells were transplanted into opposite flanks of nude mice. Control cells gave rise to xenograft tumors in 2 weeks while Ly6K knockdown cells gave rise to significantly fewer tumors. **(D)** The qRT-PCR and western blot shows Ly6E expression. **(E)** Control and Ly6E knockdown T47D cells were seeded in low dilution for colony assays. Ly6E knockdown cells have significantly reduced colony formation. **(F)** Indicated cells were transplanted into opposite flanks of nude mice previously transplanted with control-release estrogen pellets. Control cells gave rise to xenograft tumors in 3 weeks while Ly6E knockdown cells gave rise to significantly fewer tumors. The *p* values and fold change are calculated compared to control cells.

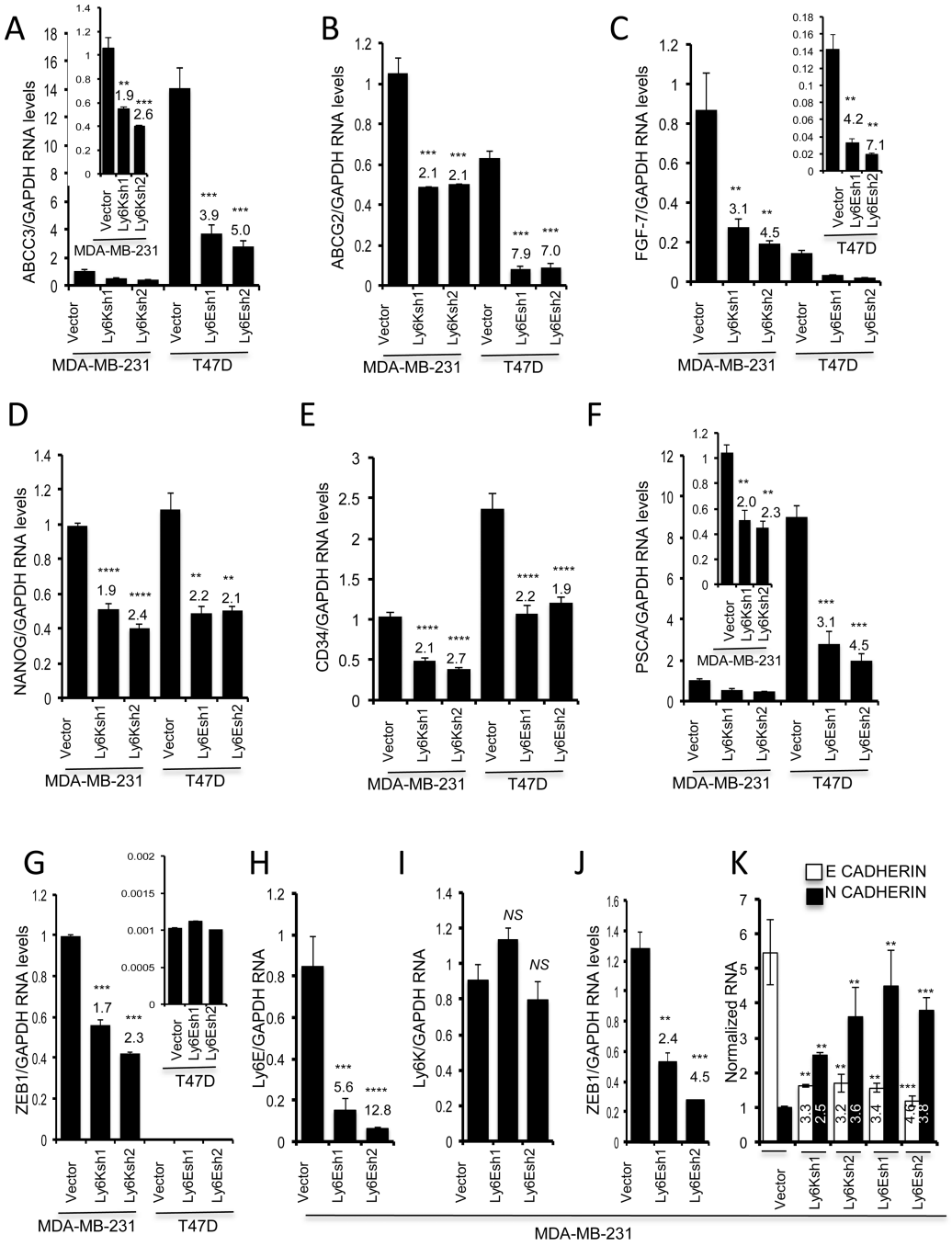

Figure 4. Ly6K and Ly6E modulates drug resistance, stem cell genes and epithelial to mesenchymal transition (EMT) pathways

Knockdown of Ly6K and Ly6E led to significantly reduced RNA levels ABCC3 **(A)** ABCG2, **(B)** and FGF-7 **(C)**, NANOG **(D)**, CD34 **(E)** and PSCA **(F)**. **(G)** The qRT-PCR revealed that Zeb1 is considerably higher in MDA-MB-231 than T47D, compare first bar in the main and inset graph. Ly6K knockdown led to reduce ZEB1, an inducer of EMT. **(H)** qRT PCR of Ly6E and **(I)** Ly6K in the indicated cells. **(J)** qRT PCR of ZEB1, **(K)** qRT PCR E-CADHERIN and N-CADHERIN. In some cases, part of a graph is presented in the inset

on a magnified scale to show the relative expressions. The *p* values are indicated by '*'. **= $p<0.05$, ***= $p<0.005$, ****= $p<0.0005$. The fold change is indicated numerically. The *p* values and fold change are calculated compared to control cells.

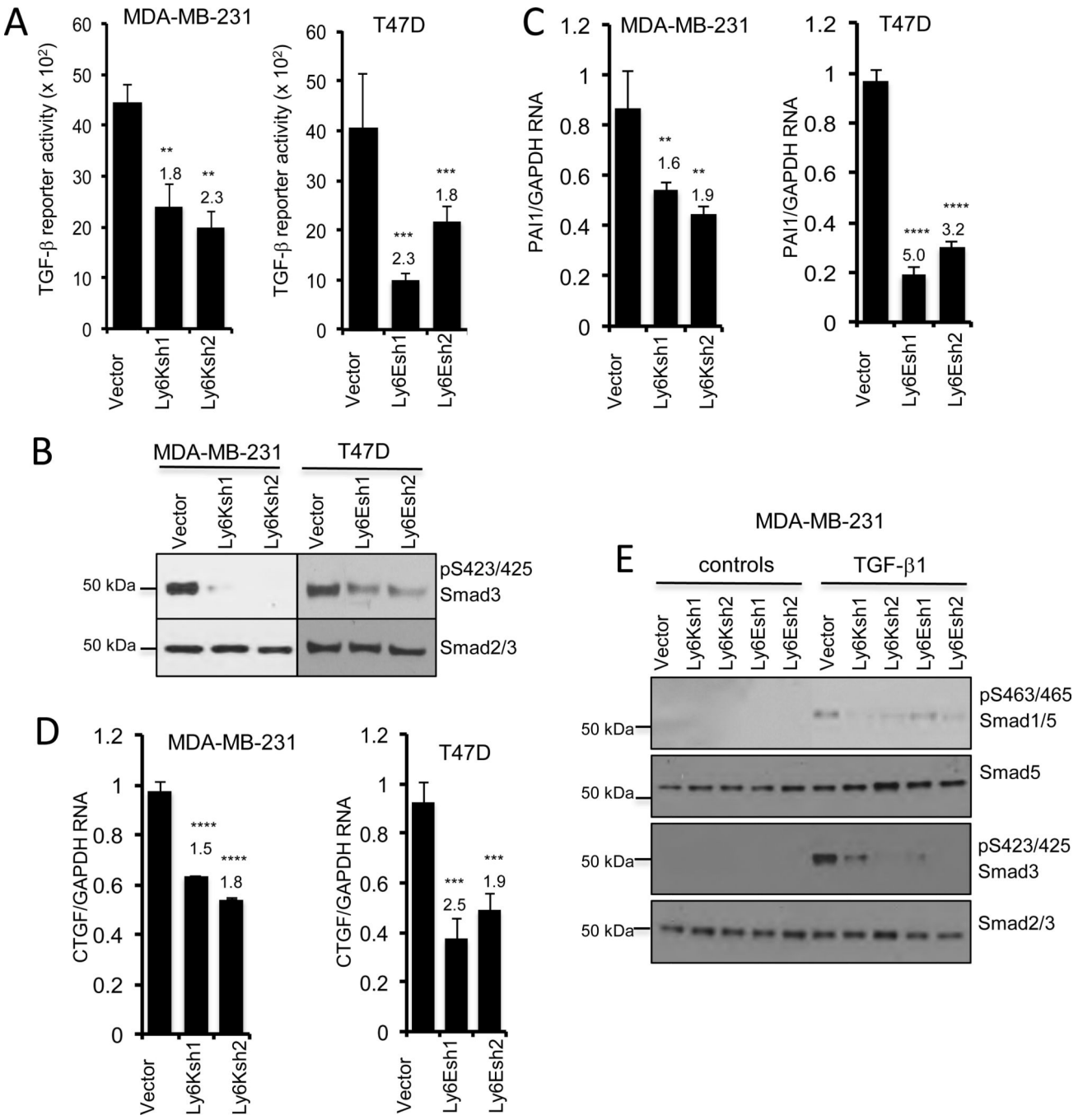

Figure 5. Ly6K and Ly6E are required for TGF-β signaling in cancer cells

(A) A luciferase reporter with Smad binding elements was transfected in the indicated cells showed that Ly6K and Ly6E knockdown cells showed significantly reduced constitutively active TGF-β signaling. **(B)** Western blotting revealed that knockdown of Ly6K and Ly6E led to reduced phosphorylation of Smad3. **(C–D)** qRT-PCR analysis shows that knockdown of Ly6K and Ly6E in led to reduced levels of well known markers of TGF-β signaling PAI1 **(C)** and CTGF **(D)**. **(E)** Western blotting revealed that ligand induced phosphorylation of Smad proteins require Ly6K and Ly6E. The *p* values are indicated by '*'. **= p<0.05,

***=p<0.005, ****=p<0.0005. The fold change is indicated numerically. The p values and fold change are calculated compared to control cells.

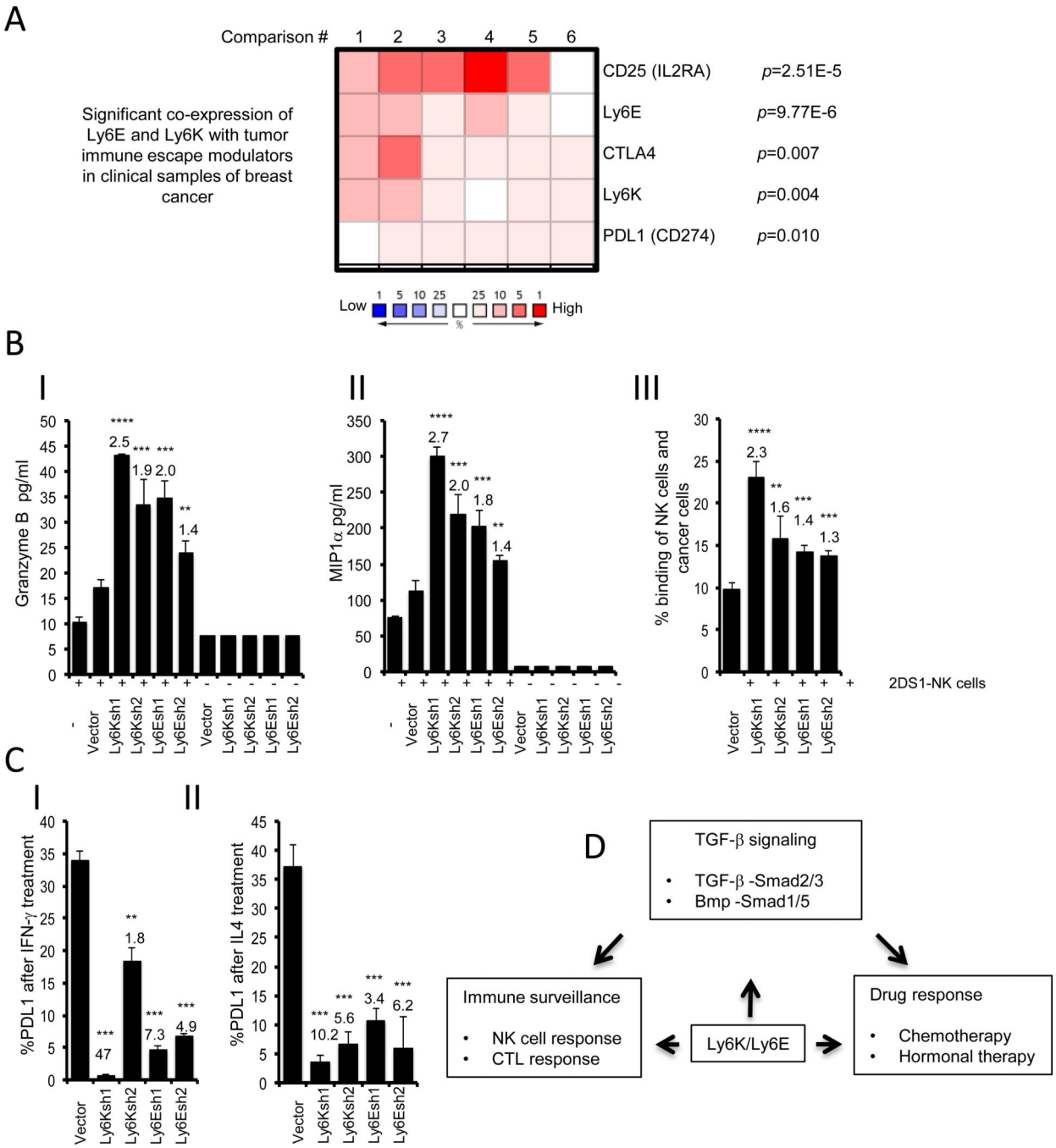

Figure 6. Ly6K and Ly6E are required for cancer cell escape from immune surveillance

(A) Significant co-expression of Ly6K and Ly6E with CD25, CTLA4 and PDL1 was observed in clinical cases of breast cancer by statistical analysis across 6 comparisons in three independent datasets (Curtis(17) **#1** TNBC n=211 vs non-TNBC n=1340, **#2** Grade1 n=89 vs Grade 3 n=857; Gluck(50) **#3** Grade 1 n=19 vs Grade 3 n=69, **#4** TNBC n=50 vs non TNBC n =101; TCGA **#5** Normal n=61 vs Invasive ductal n=389, **#6** TNBC n=46 vs non TNBC n=250) visualized by Oncomine. See Figure S5 for detailed information on each comparison. **(B)** Luminex assay using conditioned medium showed that NK cells released

significantly higher levels of Granzyme B **(I)** and MIP1α **(II)** when co-cultured with either Ly6K and Ly6E knockdown cancer cells. 2DS1 NK cells and indicated MDA-MB-231 cells were labeled with DiO and DiI cell labeling dyes, respectively, and co-cultured for 48 hours prior to flow cytometry analysis. The bar graph shows the percentage ratio of cancer cells bound to NK cells. NK cell binding was increased with Ly6K Knockdown and Ly6E knockdown cells **(III)**. **(C)** Indicated cells were treated with IFN-γ or IL4 and PDL1 surface expression was analyzed by flow cytometry. The knockdown of Ly6K or Ly6E led to reduced PDL1 expression induced by IFN-γ **(I)** or IL4 **(II)**. **(D)** Working model suggesting that Ly6K and Ly6E affect multiple aspects of cancer progression involving TGF-β signaling, drug response and tumor immune escape. The *p* values are indicated by '*'. **= $p<0.05$, ***=$p<0.005$, ****=$p<0.0005$. The fold change is indicated numerically. The *p* values and fold change are calculated compared to control cells.