



# An IL6-STAT3 loop mediates resistance to PI3K inhibitors by inducing epithelial–mesenchymal transition and cancer stem cell expansion in human breast cancer cells



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## ABSTRACT

Recently, a new generation of PI3K-specific inhibitors, such as GDC0941 and BKM120, are being investigated in clinical trials for treatment against tumors harboring *PIK3CA* mutations. Nevertheless, not all patients benefit from such treatment, suggesting that their tumors may be resistant to PI3K inhibitors. The investigation of the underlying mechanisms and efficacious personalized treatment remain a large unmet need. In this study, we revealed an IL6-STAT3 positive feedback loop that mediated the resistance to PI3K inhibitors. We found that breast cancer cells with acquired resistance to PI3K inhibitors displayed epithelial–mesenchymal transition (EMT) features and an highly enriched cancer stem cells (CSCs), secreting ~1000-fold more IL6 than parental cells. Further studies elucidated that activation of the IL6-STAT3 signaling effectively triggered EMT action, expanded the CSCs population, and reduced sensitivity to PI3K inhibitors. Pharmacological inhibition of STAT3 disrupted the IL6-STAT3 signaling and overcome resistance to PI3K inhibitors partially due to increased apoptosis induction. Taken together, our results demonstrated that feedback activation of the IL6-STAT3 loop lead to acquired resistance to PI3K inhibitors by promoting EMT and CSC-like features, and suggested that targeting this loop may be an efficient strategy to overcome resistance to PI3K inhibitors.

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## 1. Introduction

The phosphatidylinositol 3-kinase (PI3K) pathway is integral for a variety of physiological processes, including cellular growth, metabolism, differentiation, survival, and angiogenesis [1]. Mutations in components of the pathway result in constitutive activation of this signaling pathway, which are discovered in various types of cancers [2]. Recent studies have reported that mutations of *PIK3CA*, encoding the p110 $\alpha$  catalytic subunit of PI3K, occur in approximately 18% of breast cancer [3], result in constitutive activation of this signaling pathway, making it a potentially attractive therapeutic target.

Small-molecule inhibitors that target the PI3K/AKT signaling, therefore, have been developed and are currently used or being considered for the clinic. GDC0941 and BKM120, among many

others, are two novel class I PI3K inhibitors. GDC0941 have showed be efficacious in preclinical models of breast cancer [4]. Clinically, BKM120 shows good oral bioavailability and preliminary antitumor activity by specifically blocking the biologic function of PI3K signaling components, in recently completed phase I trials [5–6]. Nevertheless, not all patients benefit from GDC0941 or BKM120, even harboring the activating *PIK3CA* mutations, suggesting the development of resistance to these inhibitors. New approaches that overcome the resistance to PI3K inhibitors remain a large unmet need.

We have previously obtained two resistant cell lines (MCF7-GR and T47D-BR) derived from *PIK3CA*-mutated breast cancer cell lines (MCF7 and T47D) *in vitro* by chronic exposure to GDC0941 and BKM120, respectively, and found that these two resistant cells generated intriguing phenotypic changes consistent with epithelial–mesenchymal transition (EMT) and higher proportion of stem cell cells (CSCs) compared with parental cells. Recent evidence now indicates that, during EMT, tumor cells not only acquire potential of metastasis, but also become resistant to drugs [7]. In breast cancer, a CSCs subpopulation (CD44<sup>+</sup>CD24<sup>−</sup>) have been reported to

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contribute to resistance of chemotherapy and targeted therapy [8]. However, it is unknown if EMT and CSCs play a role in acquired resistance to PI3K inhibitors.

Here, our work proposes a novel mechanism driving resistance to PI3K inhibitors by activation of an IL6-STAT3 feedback loop leading to EMT and CSCs expansion. Notably, targeting this circuit by STAT3 inhibitor effectively recovered the sensitivity of these resistant cells to PI3K inhibitors, suggesting an effective therapeutic strategy to overcome this resistance.

## 2. Materials and methods

### 2.1. Cell lines, chemical compounds and transfection

MCF7 and T47D were obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA). MCF7-GR and T47D-BR cells were selected for resistance to the pan-p110 inhibitors GDC0941 and T47D-BR, respectively by chronic exposure to increasing concentrations of GDC0941 for ~4 months. All cell lines were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA). GDC0941, BKM120 and S31-201 (a STAT3 inhibitor) were provided by Selleck Chemicals (Houston, TX, USA). All compounds were dissolved in DMSO. Control and STAT3-specific siRNA were obtained from Santa Cruz. The transfections were done with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

### 2.2. Western blotting analysis

Whole cell lysates were prepared and Western blotting analysis was performed as described previously [9]. Primary antibodies including rabbit monoclonal antibodies against IL6 (1:1000), phospho-STAT3 (Tyr705, 1:1000), STAT3 (1:1000), phospho-AKT (Ser473, 1:1000), AKT (1:1000), phospho-ERK1/2 (Thr202/Tyr204, 1:1000), ERK1/2 (1:1000), caspase3 (1:1000) were obtained from Cell Signaling Technology (Beverly, MA, USA). Antibodies against GAPDH (1:1000) and secondary HRP-conjugated antibodies (1:4000) were purchased from CoWin Biotech (Beijing, China).

### 2.3. Real-time quantitative PCR (qPCR)

The cell total RNA was extracted using TRIzol Reagent (Invitrogen, CA, USA) according to the manufacturer's protocols, and cDNA was synthesized using a Maxima First Strand cDNA Synthesis Kit (Thermo Scientific, Rockford, IL, USA). qPCR was performed using GoTaq qPCR Master Mix with SYBR green (Promega) on Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, Foster City, USA) as recommended by the manufacturer. The screen for cytokine gene expression was performed to assess changes of resistant cells relative to parental cells using a series of primers specific for these genes. Data are shown normalized to GAPDH expression and averaged between three repeated experiments. The primer sequences are available upon request.

### 2.4. Analysis of cancer stem cells (CSCs)

Cells were collected and incubated with FITC-conjugated anti-CD44 (eBioscience, San Diego, CA, USA) and PE-conjugated anti-CD24 (eBioscience) on ice for 20 min. After washing, cells were resuspended in PBS, and analyzed using a flow cytometer (BD FACS Aria, San Jose, CA, USA).

### 2.5. Determination of cell growth and apoptosis

For cell growth analysis,  $3 \times 10^3$  cells were seeded into 96-well plates, then treated with GDC0941 (1  $\mu$ M) or BKM120 (1  $\mu$ M) for 4 days. In some experiments, recombinant IL6 (50 ng/ml) and STAT3 inhibitor S31-201 (50  $\mu$ M) were added into the medium. Cell growth was determined using CellTiter 96 AQueous One Solution Cell Proliferation Assay kit (Promega, Madison, WI, USA) according to the manufacturer's protocol.

Cell apoptosis was measured by annexin V-FITC/PI staining (BD Pharmingen). Cells ( $2 \times 10^5$ /well in 24-well plates) were treated with GDC0941 (1  $\mu$ M), BKM120 (1  $\mu$ M), S31-201 (50  $\mu$ M) or their combination for 60 h. Cells were then collected and stained with 5  $\mu$ l of annexin V-FITC and 10  $\mu$ l of PI (5 mg/ml) for 15 min in the dark. The apoptotic cells were analyzed by flow cytometry. Quantitative analyses were carried out by FlowJo software.

### 2.6. Enzyme-linked immunosorbent array (ELISA)

$1 \times 10^5$  cells were cultured in 6-well plates with RPMI 1640 containing 1% FBS for 24 h. Then the medium was completely replaced to serum-free medium for another 24 h, and the supernatant was tested using Human IL6 ELISA Kit (BioLegend, CA, USA). For some experiments, cells were transfected with control (si-Control) or STAT3-specific siRNA (si-STAT3) first, and the cytokine in the media was analyzed by ELISA.

### 2.7. Statistical analysis

The data were expressed as mean  $\pm$  s.e.m.. Differences among groups were examined by Student's *t*-test or one-way ANOVA using the GraphPad Prism program version 5 (GraphPad Software, USA). The asterisk indicates a statistically significant difference: \**p* < 0.05, \*\**p* < 0.01, \*\*\**p* < 0.001.

## 3. Results

### 3.1. PI3K inhibitor-resistant cells display characteristics of EMT and present an increased proportion of CSCs

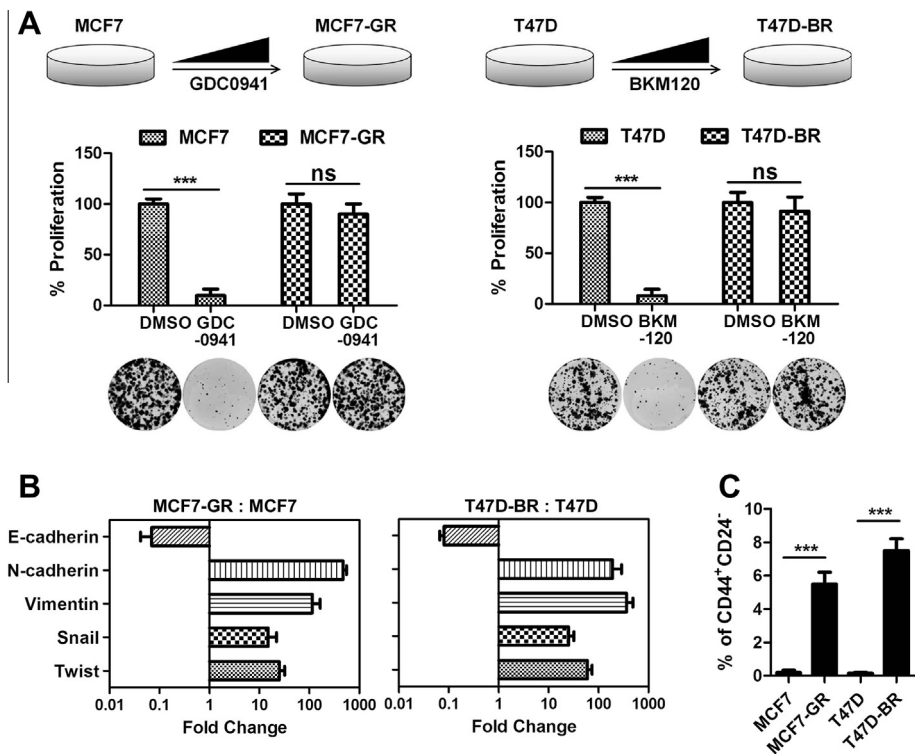
We obtained MCF7 cells with acquired resistance to GDC0941 (MCF7-GR) and T47D cells resistant to BKM120 (T47D-BR) as showed in Fig. 1A. While GDC0941 (1  $\mu$ M) and BKM120 (1  $\mu$ M) almost inhibited cell growth and clone formation of parental cells, little influences were found in their corresponding resistant cells.

Interestingly, resistant cell lines MCF7-GR and T47D-BR displayed marked changes in gene expression pattern consistent with EMT. The qPCR analysis showed a repression of E-cadherin and concomitant induction of N-cadherin, Vimentin, Snail and Twist in resistant cells compared with parental cells (Fig. 1B). Since EMT has been linked to the CSC phenotype, we examined the number of CD44<sup>+</sup>/CD24<sup>−</sup> cells, and found a significant increase in MCF7-GR and T47D-BR cells relative to their parental cells, as shown in Fig. 1C.

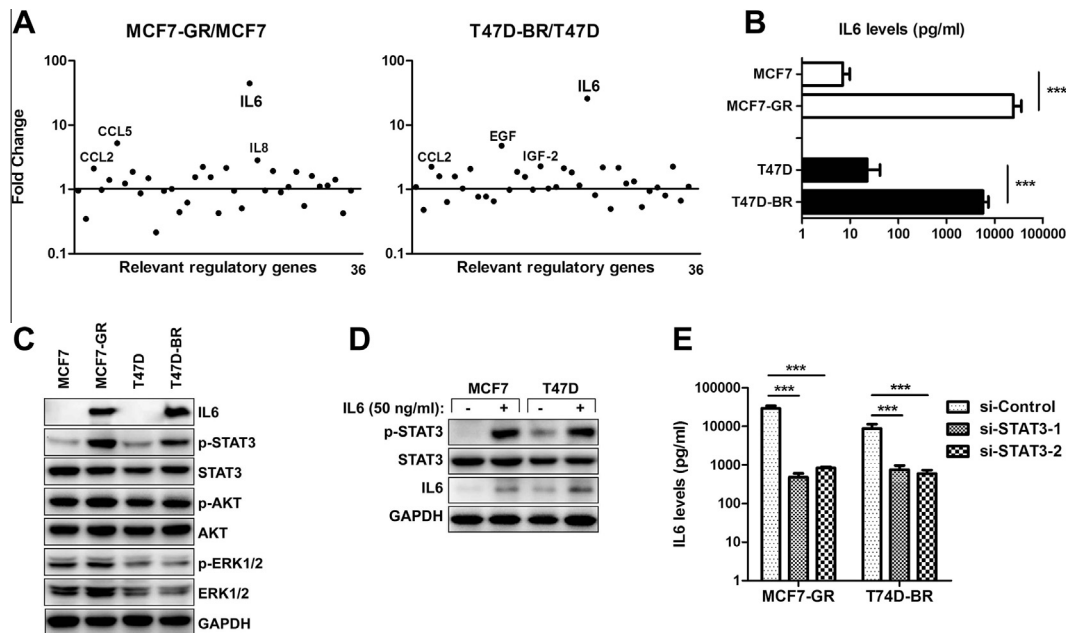
Taken together, these results elucidated that cells resistant to PI3K inhibitors were accompanied with EMT phenotype and an increased CSCs proportion.

### 3.2. An IL6/STAT3-mediated inflammatory feedback loop is activated in PI3K inhibitor-resistant cells

Taking EMT and the CSC phenotype are known to be regulated by a number of cytokines [10], we determined the levels of related cytokine expression by qPCR using specially designed primers. The results exhibited an overlapping increase of IL6 production in the



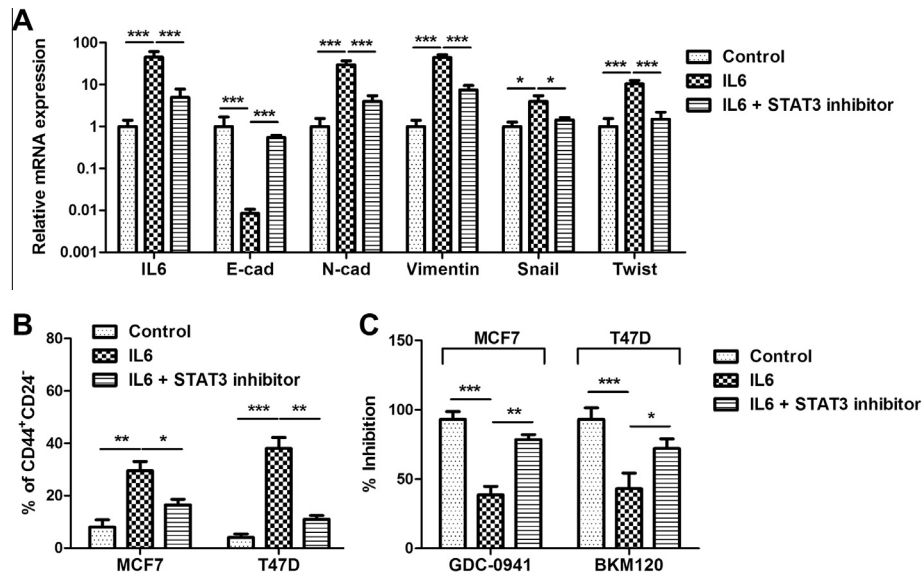
**Fig. 1.** PI3K inhibitor-resistant cells display characteristics of EMT and present an increased proportion of CSCs. (A) PI3K inhibitor-sensitive breast cancer cell lines MCF7 and T47D were made resistant by persistent exposure to increasing concentrations of GDC0941 and BKM120 (Section 3). Parental and resistant cells were cultured in the medium containing GDC0941 (1  $\mu$ M) and BKM120 (1  $\mu$ M), and cell growth and the clone capacity were measured. (B) qPCR examining the fold changes in the expression of EMT-related genes in PI3K inhibitor-resistant cells relative to parental cells. qPCR data are shown normalized to GAPDH expression and on a logarithmic scale. (C) Flow cytometry analysis of the proportions of CD44<sup>+</sup>CD24<sup>-</sup> cells in parental and corresponding resistant cells.



**Fig. 2.** An IL6/STAT3-mediated inflammatory feedback loop is activated in PI3K inhibitor-resistant cells. (A) Scatter plot of fold-change of 33 cytokines genes and 3 housekeeper- or control-genes in PI3K inhibitor-resistant versus parental cell lines. (B) The level of IL6 in the culture supernatants was examined by ELISA. (C) Western blotting evaluating major cell signaling changes in the indicated PI3K inhibitor-sensitive and -resistant cells. (D) Cells were treated with recombinant IL6 (50 ng/ml) for 24 h, and the STAT3 activity and IL6 expression were detected by Western blotting. (E) After transfection with control (si-Control) or STAT3-specific siRNA (si-STAT3), the cell supernatants were assayed for IL6 by ELISA. Data are means  $\pm$  s.e.m. from three independent experiments. \*\*\* $p$  < 0.001 for the indicated comparisons.

two PI3K inhibitor-resistant cells, compared with their corresponding parental cells (Fig. 2A). A slight up-regulation of CCL2, CCL5 and IL8 in MCF7-GR cells and a mild elevated CCL2, EGF and IGF-2 in T47D-BR cells were also observed, but the changes

were individual phenomena or did not reach statistical significance (data not shown). Utilizing an ELISA, we further confirmed that there was  $\sim$ 1000-fold increase in the secretion of IL6 in resistant cells compared with parental cells (Fig. 2B). This dramatic



**Fig. 3.** IL6 induces EMT, expands the CSCs population and mediates resistance to PI3K inhibitors through STAT3 pathway. (A) MCF7 cells were stimulated with recombinant IL6 (50 ng/ml) in the presence or absence of STAT3 inhibitor S3I-201 (50  $\mu$ M), then the relative mRNA levels of IL6 and EMT-related genes were examined by qPCR. (B) Flow cytometry analysis of the proportions of CD44<sup>+</sup>CD24<sup>-</sup> cells in MCF7 and T47D cells after treatment with IL6 (50 ng/ml) and STAT3 inhibitor S3I-201 (50  $\mu$ M). (C) Effects of IL6 stimulation (50 ng/ml) and STAT3 inhibition (S3I-201; 50  $\mu$ M) on PI3K inhibitor-mediated cell growth inhibition. Data are means  $\pm$  s.e.m. from three independent experiments. \* $p$  < 0.05, \*\* $p$  < 0.01 and \*\*\* $p$  < 0.001 for the indicated comparisons.

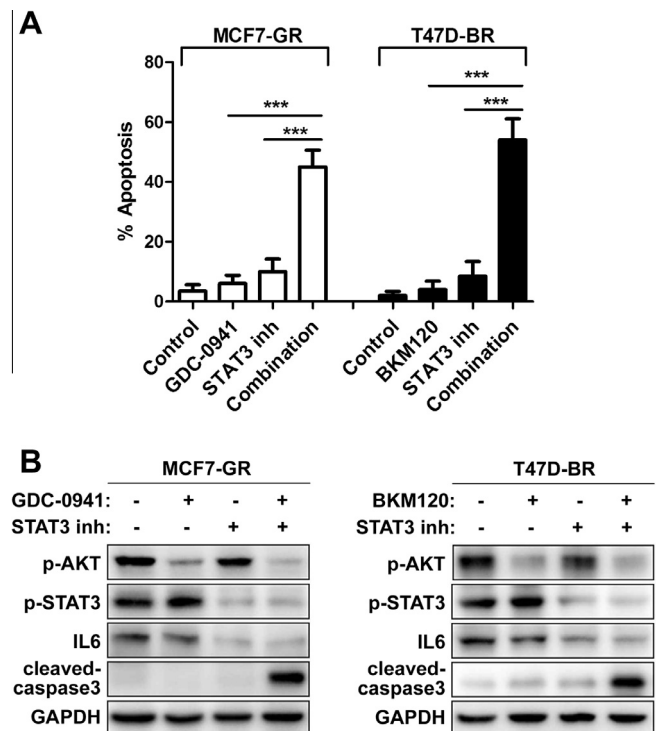
elevation of IL6, may be involved in drug resistance as well as regulation the EMT and CSC-like properties in PI3K inhibitor-resistant cells.

The changes in main cell signaling transduction pathways were further examined. While the expression of IL6 was elevated in MCF7-GR and T47D-BR cells consistent with the data above, a significant increase of STAT3 activity was detected in both PI3K inhibitor-resistant cells relative to the corresponding parental cells (Fig. 2C). STAT3 signaling pathway is reported to transcribe a number of cytokine genes including IL6 [11]; conversely, IL6 can also activate STAT3 signaling, constituting a positive feedback loop [12]. Consistent with these studies, stimulation with recombinant IL6 markedly induced STAT3 phosphorylation as well as the expression of IL6 itself in parental cells as shown by Western blotting (Fig. 2D). We utilized STAT3-specific siRNA to determine the effect on IL6 production in MCF7-GR and T47D-BR cells, and found that STAT3 depletion completely suppressed the secretion of IL6 (Fig. 2E), indicating the regulation of IL6 by STAT3. Together, cytokines IL6 and STAT3 maintained a positive feedback loop, which was permanently activated in cells resistant to PI3K inhibitors.

### 3.3. IL6 induces EMT, expands the CSCs population and mediates resistance to PI3K inhibitors through STAT3 pathway

Previous studies have reported the induction of EMT and stem cells-like properties by IL6 in breast cancer cells [13–14]. We further elucidated whether the IL6-STAT3 signaling contributed to these processes in our model. Stimulation with IL6 enhanced the mRNA levels of itself, and also resulted in E-cadherin (E-cad) repression and up-regulation of N-cadherin (N-cad), Vimentin, Snail and Twist. STAT3-specific inhibitor was able to reverse such changes (Fig. 3A). We further examined whether the IL6 could render the expansion of CSCs population in parental MCF7 cells *in vitro*. Indeed, IL6 significantly increased the number of CD44<sup>+</sup>/CD24<sup>-</sup> cells, which were further abolished by STAT3 inhibition. Notably, IL6 stimulation markedly impaired the sensitivity to PI3K inhibitors, suggesting that IL6 mediated the resistance to PI3K inhibitors by inducing EMT and CSC features. Moreover,

IL6-induced resistance was almost abolished by STAT3 inhibition, indicating the pivotal role of IL6-STAT3 pathway in this progress.



**Fig. 4.** STAT3 inhibition overcomes resistance to PI3K inhibitors. (A) Cells were treated with GDC0941 (1  $\mu$ M), BKM120 (1  $\mu$ M), STAT3 inhibitor (50  $\mu$ M S3I-201) or combined treatment in MCF7-GR and T47D-BR cells for 60 h, then the frequency of apoptosis was examined by annexin V-FITC/PI staining. The apoptosis cells contained the subpopulation of cells staining with annexin V<sup>+</sup>/PI<sup>-</sup> and annexin V<sup>+</sup>/PI<sup>+</sup>. (B) Western blotting evaluating AKT and STAT3 activities as well as the expression of IL6 and cleaved-caspase3 in response to GDC0941 (1  $\mu$ M), BKM120 (1  $\mu$ M), STAT3 inhibitor (50  $\mu$ M S3I-201) or combined treatment in MCF7-GR (left) and T47D-BR cells (right). Data are means  $\pm$  s.e.m. from three independent experiments. \*\*\* $p$  < 0.001 for the indicated comparisons.



Together, these results suggested that activation of IL6-STAT3 signaling was capable of inducing resistance to PI3K inhibitors, as well as accompanying EMT and CSC-like phenotypes in breast cancer cells.

### 3.4. STAT3 inhibition overcomes resistance to PI3K inhibitors

PI3K inhibitors alone failed to elicit cell apoptosis (Fig. 4A), although phosphorylated AKT was almost abrogated in MCF7-GR and T47D-BR cells (Fig. 4B). Notably, when used in combination with S3I-201 (a STAT3 inhibitor), GDC-0941 and BKM120 caused a prominent apoptosis in both resistant cells. These results were further validated by Western blotting that combination treatment suppressed both STAT3 and AKT activities and induced obvious induction of cleaved-caspase3, a marker for cells undergoing apoptosis. Additionally, the expression of IL6 was abolished after inhibition of STAT3 activity, consistent with the results shown in Fig. 2E.

Altogether, these results demonstrated that inhibition of STAT3 recovered the sensitivity to PI3K inhibitors at least partially due to increased apoptosis induction, and dual inhibition of PI3K and STAT3 may be an effective strategy to overcome resistance to PI3K inhibitors in this scenario.

## 4. Discussion

Mutations of *PIK3CA* occur commonly in many different cancers, leading to dysregulation of PI3K signaling [15]. Given the pivotal role of this pathway in tumor progression, numerous evidences suggested that *PIK3CA* mutations may be the predictor for response to PI3K inhibitors [16]. However, not all tumor cells carrying the *PIK3CA*-activating mutations are sensitive to PI3K pathway inhibitors in preclinical and clinical studies. The experience with previous targeted therapy paradigms suggests that primary or acquired resistance is a major limitation with these small-molecule inhibitors. The identification of underlying mechanisms triggering the resistance to PI3K inhibitors could furnish the design of successful strategies to circumvent them.

In this study, we established two PI3K inhibitor-resistant breast cancer cells (MCF7-GR and T47D-BR) by chronically exposing the parental cells to increasing dose of GDC0941 and BKM120 respectively for a long period of time *in vitro*. Cloning formation assay showed that GDC0941 and BKM120 failed to inhibited the cell growth of MCF7-GR and T47D-BR, respectively, while parental MCF7 and T47D cells harboring activating *PIK3CA* mutations, were selectively sensitive to the PI3K inhibitors *in vitro* (Fig. 1A). At this concentration (1  $\mu$ M) of GDC0941 or BKM120, phosphorylated AKT was almost abrogated in both resistant cells (as shown in Fig. 4B), suggesting that resistance was not due to lack of target inhibition.

More interestingly, cells resistant to PI3K inhibitors displayed characteristics of EMT and exhibited stem cell-like features (CSCs). EMT and CSCs are known to contribute to tumor growth and metastasis, and may be responsible for drug resistance [17]. It has reported that decreased E-cadherin expression, a hallmark of EMT, is associated with resistance to EGFR kinase inhibitors, with drug-sensitive cells being more epithelial-like [18]. And in epithelial-like luminal breast cancers with HER2/neu amplification, cells with CSC features (CD44<sup>+</sup>CD24<sup>-</sup>) are resistant to chemotherapy or lapatinib [19]. Moreover, disseminated breast cancer cells that have presumably undergone EMT, may exhibit a phenotype of CD44<sup>+</sup>CD24<sup>-</sup> cells, connecting EMT to the emergence of stem cells [8]. These results therefore suggested that, in our case, induction of EMT and the emergence of CSCs may also be involved in the resistance to PI3K inhibitors.

A diverse array of cytokines and growth factors are reported to induce EMT and CSCs properties [10], and likely represent a key

convergence point in the mechanism of resistance to PI3K inhibitors. By comparing the changes of a number of reported factors that regulate EMT and CSCs, we found an overlapped increase in IL6 production (Fig. 2A), which was further validated by ELISA (Fig. 2B). High levels of IL6 have been detected in breast tumor tissues and blood serum, and correlate with advanced tumor grades, increased metastatic propensity, and poor survival [20]. Recent studies have elucidated that IL6 is capable to initiate EMT in human breast cancer cells via activating STAT3-Twist signaling [13]. IL6 is also considered to expand the CSC population in breast cancer cells [14]. Such that IL6 appears to be the key factor that are responsible for the resistance to PI3K inhibitors in breast cancer cells with EMT and CSC traits. Here, addition of recombinant IL6 in parental cells lead to the expression of EMT-associated genes (Fig. 3A) and CSCs expansion (Fig. 3B), as well as desensitivity to PI3K inhibitors treatment (Fig. 3C). Thus, these results indicated that breast cancer cells acquired resistance to PI3K inhibitors through up-regulating IL6 production, and subsequent induction of EMT and CSC features. Additionally, although IL6 induced a 4–10 folds increase in the CD44<sup>+</sup>CD24<sup>-</sup> cells, it is unclear whether this increase are due to the expansion of the already existing content of CSCs or generation from non-CSC subpopulation. A recent study suggested that IL6 enriched the CD44<sup>+</sup> cell population by up-regulating CD44 protein expression of non-CSC. Further studies will be necessary to investigate this possibility in our system.

Our data showed that striking activation of STAT3, but not AKT or ERK, was detected in both PI3K inhibitor-resistant cells with accompanying IL6 up-regulation (Fig. 2C), consistent with previous studies suggested that activation of STAT3 signaling was involved in induction of EMT and CSCs attributes [9,21]. Notably, recombinant IL6 effectively induced STAT3 phosphorylation as well as the up-regulation of IL6 itself in parental cells (Fig. 2D); conversely, depletion of STAT3 markedly decreased the production of IL6 in both PI3K inhibitor-resistant cells (Fig. 2E), indicating the regulation of IL6 by STAT3 in our system. These results therefore indicated that an IL6-STAT3 signaling loop was activated in cells resistant to PI3K inhibitors, and may be responsible for the generation of EMT and CSC features. Indeed, targeting STAT3 with a small-molecule inhibitor effectively abolished IL6-induced EMT characteristics, and increase of CSC subpopulation (Fig. 3A and B).

Emerging evidence suggests that the feedback loop based on IL6 and STAT3 contribute to resistance of various drugs, and could be presented as an effective targets [22,23]. Similarly, in our system, IL6 induced STAT3 activation and subsequently decreased the sensitivity of parental cells to PI3K inhibition, while pharmacological inhibition of STAT3 disrupted this feedback loop and recovered the sensitivity to PI3K inhibitors, suggesting the pivotal role of STAT3 pathway in regulation the resistance to PI3K inhibitors. Furthermore, while inhibitors that targeting PI3K or STAT3 alone cannot elicit obvious apoptosis in PI3K inhibitor-resistant cells, dual inhibition of PI3K and STAT3 triggered a significant frequency of apoptosis than each agent alone (Fig. 4A), indicating that targeting STAT3 can overcome PI3K inhibitor resistance partially due to increased apoptosis induction.

In conclusion, our work proposed a novel mechanism of IL6-STAT3 feedback loop-mediated resistance of PI3K inhibitor via induction of EMT and CSC traits. These tumors may be exquisitely sensitive to STAT3 inhibitors, providing the rationale for innovative therapeutic strategies.

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