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Repression of Smad3 by Stat3 and c-Ski/SnoN induces gefitinib resistance in lung adenocarcinoma

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ABSTRACT

Cancer-associated inflammation develops resistance to the epidermal growth-factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) in non-small cell lung cancers (NSCLCs) harboring oncogenic EGFR mutations. Stat3-mediated interleukin (IL)-6 signaling and Smad-mediated transforming growth factor-β (TGF-β) signaling pathways play crucial regulatory roles in cancer-associated inflammation. However, mechanisms how these pathways regulate sensitivity and resistance to EGFR-TKI in NSCLCs remain largely undetermined. Here we show that signal transducer and activator of transcription (Stat)3 represses Smad3 in synergy with the potent negative regulators of TGF-β signaling, c-Ski and SnoN, whereby renders gefitinib-sensitive HCC827 cells resistant. We found that IL-6 signaling via phosphorylated Stat3 induced gefitinib resistance as repressing transcription of Smad3, whereas TGF-β enhanced gefitinib sensitivity as activating transcription of Smad3 in HCC827 cells with gefitinib-sensitizing EGFR mutation. Promoter analyses showed that Stat3 synergized with c-Ski/SnoN to repress Smad2/3/4-induced transcription of the *Smad3* gene. Smad3 was found to be an apoptosis inducer, which upregulated pro-apoptotic genes such as caspase-3 and downregulated anti-apoptotic genes such as Bcl-2. Our results suggest that derepression of Smad3 can be a therapeutic strategy to prevent gefitinib-resistance in NSCLCs with gefitinib-sensitizing EGFR mutation.

1. Introduction

Although EGFR-TKIs have remarkably improved the therapeutic efficacy for NSCLCs harboring the sensitive EGFR mutation [1], drug resistance inevitably develops [2]. Genetic changes such as secondary mutations in EGFR (T790M) and amplification of the MET receptor tyrosine kinase are the main mechanisms of acquired drug resistance [3,4]. Unknown mechanisms of EGFR-TKI resistance in the remaining cases [4] could be attributed to chronic inflammation, which plays critical roles in tumorigenesis [5]. It is also possible that chronic inflammatory responses induce DNA damages and mutations [6].

IL-6 is the pivotal pro-inflammatory cytokine in inflammation including cancer-associated inflammation [7,8], which affects treatment effectiveness [9]. IL-6 and EGF share Stat3 for their signaling pathways, which promote carcinogenesis through inflammation [10,11]. In NSCLCs, correlation between elevation of IL-6 and phosphorylation of Stat3 are significantly correlated [12,13].

In contrast with IL-6, TGF-β is the principal immunosuppressive cytokine secreted in the tumor microenvironment [14]. Elevation of TGF-β is frequently observed in NSCLCs, which is correlated with

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Abbreviations

EGFR	epidermal growth-factor receptor
TKIs	tyrosine kinase inhibitors
NSCLCs	non-small cell lung cancers
TGF-β	transforming growth factor-β
IL-6	interleukin-6
STAT	signal transducer and activator of transcription

poor prognosis and metastasis [15]. Cytoplasmic proteins called Smads mediate the canonical pathway for signal transduction from the TGF-β receptor (TβR) serine threonine kinases. Activated TβRI phosphorylates serine residues in MH2 domains of receptor-regulated Smads (R-Smads), Smad2 and Smad3, which oligomerize with the common-mediator Smad, Smad4 to regulate transcription of the target genes [16,17]. Smad2 and Smad3 are highly homologous molecules with distinct effects in context-dependent manners, however, detailed mechanisms of involvement of Smad2 versus Smad3 in carcinogenesis remains largely undetermined [18].

Transmodulation between the STAT and SMAD signaling pathways has been implicated as the basis for the antagonism between TGF-β and pro-inflammatory cytokines such as interferon-γ, IL-1β and TNF-α [19,20]. It has been reported that TGF-β-dependent IL-6 secretion and subsequent Stat3 activation induce EGFR-TKI resistance in NSCLCs [21]. We previously reported that Smad2 and Smad3 oppositely regulate Th17 differentiation as Stat3 cofactors [22]. However, the mechanisms whether and how Stat3-mediated IL-6 signaling and Smad-mediated TGF-β signaling interact to regulate EGFR-TKI resistance in NSCLCs remain largely unknown.

In this study, we examined the mechanisms how R-Smads and Stat3 regulate the sensitivity and resistance to gefitinib in HCC827 lung adenocarcinoma cells with gefitinib-sensitizing EGFR mutation.

2. Materials and methods

2.1. Cell culture and transfection

Human lung adenocarcinoma HCC827 cells (ATCC) were cultured in RPMI 1640 (Hyclone) supplemented with 10% heat-inactivated FBS (Gibco) and 1% penicillin/streptomycin (ThermoScientific) with or without the combinations of human IL-6 (R&D, 10 ng/ml), TGF-β1 (R&D, 5 ng/ml) and gefitinib (AstraZeneca, 2 μM) for 4 h after serum starvation and grown at 37 °C with 5% CO_2. No mutations or copy number variations of TGF-β signaling molecules are observed in HCC827 cells (http://cancer.sanger.ac.uk/cell_lines/sample/overview?id=1240146). HCC827 cells were seeded a day before transfection and transfected with Smad3 cDNA, Smad3 siRNA, Stat3 siRNA or control siRNA (GE Dharmacon) using Lipofectamine LTX & Plus Reagent (Invitrogen) following manufacture's protocol as previously described [22].

2.2. Cell cycle analysis

DNA content was determined by propidium iodide (PI; BD Pharmingen). Briefly, HCC827 cells were fixed by cold 80% ethanol overnight at −20 °C, washed twice with phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA), and then resuspended in PI solution in PBS containing RNase A (0.1 mg/ml; Sigma-Aldrich). Stained samples were acquired by FACSCalibur (BD Bioscience) and analyzed by FlowJo (Tree Star).

2.3. Immunocytochemistry

Proximity ligation assay (PLA) was performed as previously described [22]. HCC827 cells were fixed with 4% formaldehyde. Fixed slides were permeabilized by 0.1% Triton X-100 in PBS for staining with Duolink in situ PLA probes anti-rabbit PLUS and PLA probe anti-rabbit MINUS or PLA probe anti-mouse PLUS and PLA probe anti-mouse MINUS and detection reagents (Sigma-Aldrich), rabbit or mouse antibodies against Smad2, Smad3, phospho-Stat3 Y705, phospho-Stat3 S727 (Cell Signaling Technology), Stat3, c-Ski and SnoN (Santa Cruz). Nucleus was stained with DAPI. Slides were observed using a confocal microscope, LSM700 (Carl Zeiss). PLA signals were quantified using BlobFinder software (http://www.cb.uu.se/~amin/BlobFinder/).

2.4. RNA isolation and quantitative RT-PCR

Total RNA was extracted from HCC827 cells using Trizol according to the manufacturer's instructions (Invitrogen). RNA was reverse transcribed with a cDNA RT kit (Invitrogen). Human cDNA was quantitated by SYBR green (Applied Biosystems) using ABI 7900 (Applied Biosystems). Each experiment was performed in triplicate. The following primers were used: GAPDH 5′- CTCAAGACCTTGGGCTGGG-3′, 5′-TCGAACAGGAGGAGCAGAGA-3′, Smad3 5′-GAGTTGAGGCGAAGTTTGGG-3′, 5′-AGTGAAAGGCAGGATGGACG-3′, Bcl2 5′-AACATCGCCCTGTGGATGAC-3′, 5′-CAGGGCCAAACTGAGCAGAG-3′, Bbc3 5′-ACTCACCAAACCAGAGCAGG-3′, 5′-ACAAATCTGGCAGGGGACC-3′, Bnipl 5′-AACCAGCTCCCCAACAACTC-3′, 5′-ATGTCTCAGGGCTGCTCCTA-3′, Caspase2 5′-CTTGCTGCCTAAGAGGGGTC-3′, 5′- CTCACACACCGGAAAAGGGA-3′, Caspase3 5′-CTCTGGTTTTCGGTGGGTGT-3′, 5′-TCCAGAGTCCATTGATTCGCT-3′, Cidec3 5′-CAGAGCCAGGGGATGAGAAA-3′, 5′-CACAGAGGTACGCACTGACA-3′

2.5. Luciferase reporter assay

The 2000 bp promoter region of Smad3 was generated by PCR from genomic DNA using the primers: 5′-GATCACGCGTTTGGGTTCAAATTCCAGCTC-3′ and 5′-GATCCTCGAGGCAGCAGAAGTTTGGGTTTC-3′. The product was verified by sequencing and was subcloned into pGL3 Basic firefly luciferase construct (Promega) using Mlu1 and *Xho*I sites. The promoter construct was transfected in various combination with Stat3, Flag-tagged Stat3Y705F, Stat3 S727A (Addgene, submitted by J. Darnell), Flag-tagged Smad2/3/4, Flag-tagged c-Ski, SnoN, TGIF or empty pcDNA3 plasmid in HCC827 cells using Lipofectamine (Invitrogen). Twenty-four hours after transfection, HCC827 cells were lysed using lysis buffer for the measurement using luciferase assay kit (Promega) by luminometer. Each experiment was performed in triplicate.

2.6. Chromatin immunoprecipitation (ChIP)

Chromatin was prepared from HCC827 cells. Immunoprecipitation was performed with antibodies against Smad2, Smad3, trimethyl histone H3 Lys4 and tri-methyl histone H3 Lys27 (Cell Signaling Technology), Smad4, c-Ski, SnoN and STAT3 (Santa Cruz) using ChIP kit (Cell Signaling Technology) according to the manufacturer's protocol. Immunoprecipitated DNA released from the cross-linked proteins was quantitated by real time PCR using the primers: −1700 to −1534 5′-AATGCCAAGTAAGGCACAGG-3′ 5′-CTCCCTTCCACTTGCTGCTA-3′, −1559 to −1410 5′-TCTCTAGCAGCAAGTGGAAGG-3′ 5′-GCAGCTTGTCAGGGGTTGT-3′, −1196 to −1003 5′-TAGCCTGATAGGGAGGCTGA-3′ 5′-CCGGAGAGGACTCGAGAAGT-3′, −524 to −372 5′-GAGCTTTTCTGAACCCCTCA-3′ 5′-ACCGGACTCCTGGGGACT-3′, −220 to −28 5′-

CTGGGAAGGAGGCTGCAC-3′ 5′-AAACTTTGCTGGCCTGGAT-3′ −107 to +63 5′-CAGAGGAGGAGGAGGAGGAG-3′ 5′-CGAGACTCCAAGTGGCAGTAG-3′ and was normalized to input DNA. Each experiment was performed in triplicate.

3. Results

3.1. Stat3-mediated IL-6 signaling induces gefitinib resistance in HCC827 lung adenocarcinoma cells

We used HCC827 lung adenocarcinoma cells with EGFR746E-750A deletion to examine the mechanisms how IL-6 and its downstream Stat3 signaling induce gefitinib resistance in this study. TGF-β and gefitinib induced apoptosis with synergistic pro-apoptotic effect, whereas IL-6 suppressed TGF-β/gefitinib-induced apoptosis (Fig. 1A). IL-6 enhanced phosphorylation of Stat3 at tyrosine 705 (pY705) and serine 727 (pS727), whereas TGF-β and gefitinib suppressed Stat3 phosphorylation (Fig. 1B). Consistent with the previous report [23], we confirmed that Stat3 deletion by RNAi accelerated, whereas Stat3 overexpression inhibited TGF-β/gefitinib-induced apoptosis (Fig. 1C, Supplementary Fig. 1). Thus, Stat3-mediated IL-6 signaling induced resistance to TGF-β/gefitinib in HCC827 cells.

3.2. Stat3-mediated IL-6 signaling represses Smad3 in HCC827 cells

We next examined the status of TGF-β receptor-regulated Smads (R-Smads), Smad2 and Smad3 in HCC827 cells treated with TGF-β and gefitinib with or without IL-6. We found that IL-6 abolished the expression of Smad3 protein, whereas Smad2 remained expressed (Fig. 2A). IL-6 completely inhibited the expression of Smad3 mRNA (Fig. 2B), indicating that IL-6 repressed the transcription of the *Smad3* gene. By contrast, TGF-β and gefitinib upregulated Smad3 mRNA (Fig. 2B). Luciferase reporter spanning 2 kilobase upstream of the first exons of the *Smad3* genes showed that IL-6 repressed, whereas TGF-β and gefinitib induced the *Smad3* gene promoter activity (Fig. 2C). Canonical TGF-β signaling molecules, R-Smads: Smad2/3 and common Smad: Smad4 synergistically induced the *Smad3* gene promoter activity (Fig. 2D). By contrast, Stat3 repressed the Smad3/4-induced *Smad3* gene promoter activity (Fig. 2E). Consistently with the phosphorylation status of Stat3 (Fig. 1B) and the expression levels of Smad3 (Fig. 2A), Stat3 mutations at Y705 or S727 completely abolished this repression (Fig. 2E), indicating that Stat3 phosphorylation at Y705 and S727 is essential for Stat3-induced repression of the *Smad3* gene. Overexpression of Stat3 significantly suppressed, whereas Stat3 knockdown upregulated the expression of Smad3 protein in HCC827 cells (Fig. 2F). These results show that Smad-mediated TGF-β signaling induces, whereas Stat3-mediated IL-6 signaling represses the transcription of the *Smad3* gene.

3.3. c-Ski and SnoN cooperate with Stat3 to repress Smad3

We sought to determine how Stat3 represses the *Smad3* gene. We screened the representative Smad co-repressors: Ski/SnoN and TGIF [17,24–26] for Stat3 co-repressors, and found that c-Ski and SnoN, but not TGIF showed synergy with Stat3 to repress the Smad3/4-induced *Smad3* gene promoter activity (Fig. 3A). c-Ski and SnoN are the potent negative regulators of TGF-β signaling [24–26]. ChIP showed that Smad2/3/4 were bound to the same site in the *Smad3* promoter (−1700 to −1534) upon TGF-β stimulation (Fig. 3B, middle), which was epigenetically active with methylated histone H3 lysine 4 (Fig. 3C, left). By contrast, Stat3, c-Ski and SnoN were bound to the *Smad3* promoter (−1559 to −1410), overlapped or proximal to the Smad2/3/4 biding site upon IL-6 stimulation (Fig. 3B, lower), which was epigenetically inactive with trimethylated histone H3 lysine 27 (Fig. 3C, right). PLA showed that TGF-β inhibited, whereas IL-6 enhanced the interactions between Stat3 and c-Ski/SnoN (Fig. 3D) in accordance with Stat3 phosphorylation status (Fig. 1B). These results indicate that phosphorylated Stat3 cooperates with c-Ski and SnoN to repress Smad-induced transcription of the *Smad3* gene.

3.4. Smad3 induces apoptosis and restores IL-6-induced gefitinib resistance

We examined the physiological relevance of IL-6/Stat3-induced repression of Smad3 to gefitinib resistance by overexpression or knockdown of Smad3 in HCC827 cells. Forced expression of Smad3 significantly induced, whereas knockdown of Smad3 by RNAi significantly inhibited spontaneous and gefitinib/TGF-β-induced apoptosis of HCC827 cells (Fig. 4A). IL-6-induced gefitinib resistance was completely abolished by forced expression of Smad3 (Fig. 4A, right third row). Smad3 overexpression upregulated pro-apoptotic genes such as *Caspase3*, *Caspase2* and *Cidec* (cell death-inducing DNA fragmentation factor-like effector c), while repressed anti-apoptotic genes such as *Bcl2* (b-cell leukemia/lymphoma 2 protein), *Bbc3* (BCL2 Binding Component 3) and *Bnipl* (BCL2 Interacting Protein Like) (Fig. 4B). By contrast, Smad3 knockdown showed the opposing patterns (Fig. 4B). These data show that Smad3 enhances gefitinib/TGF-β-induced apoptosis and restores IL-6-induced gefitinib resistance by upregulating pro-apoptotic genes and downregulating anti-apoptotic genes in HCC827 cells.

4. Discussion

Various mechanisms lead to inevitable development of EGFR TKI resistance including the secondary mutations to a drug-resistant state, activation of alternative signaling pathways, impairment of EGFR-TKI-mediated apoptosis and histologic transformation or epithelial-mesenchymal transition (EMT) [27]. Chronic inflammatory responses in tumor microenvironment not only induce drug resistance but also induce DNA damages and mutations [6,21,23]. Thus, it is important to investigate the inflammatory mechanisms to induce EGFR TKI resistance. The representative pro-inflammatory cytokine in cancer-associated inflammation, IL-6 is the potent inducer of EGFR TKI resistance in NSCLCs [23]. TGF-β is essential for erlotinib resistance, EMT and increased activation of IL-6 axis in resistant H1650 cells [21]. Unlike resistant NSCLCs, TGF-β exerted synergistic pro-apoptotic effect with gefitinib in case of highly EGFR TKI-sensitive HCC827 cells [28], in which both TGF-β and gefitinib suppressed Stat3 phosphorylation at Y705 and S727 in the absence of IL-6. The most significant difference between highly EGFR TKI-sensitive HCC827 cells and resistant NSCLC cell lines is IL-6-induced repression of Smad3 in HCC827 cells. By contrast, Smad3 remained expressed and highly phosphorylated in resistant H1650 cells with augmented IL-6 secretion [23]. It has been reported that high expression of Smad3 and profilin-2 is linked to poor survival rate in lung cancer patients [29]. Because TGF-β switches from tumor suppressor at early stages of carcinogenesis to pro-oncogene at later stages of disease leading to metastasis [16], our findings suggest that combined anti-inflammatory therapies with EGFR-TKI at early stages may prevent cancer progression.

Although aberrant TGF-β signaling such as the mutations/deletion of Smad4 and the TGF-β type II receptor is frequent in cancers, the mutations/deletion of Smad2 and Smad3 are rarely found [30]. Loss of Smad2 or Smad3 by post-transcriptional modulations has been reported in various cancers [18]. However, precise molecular mechanisms how their expression is selectively

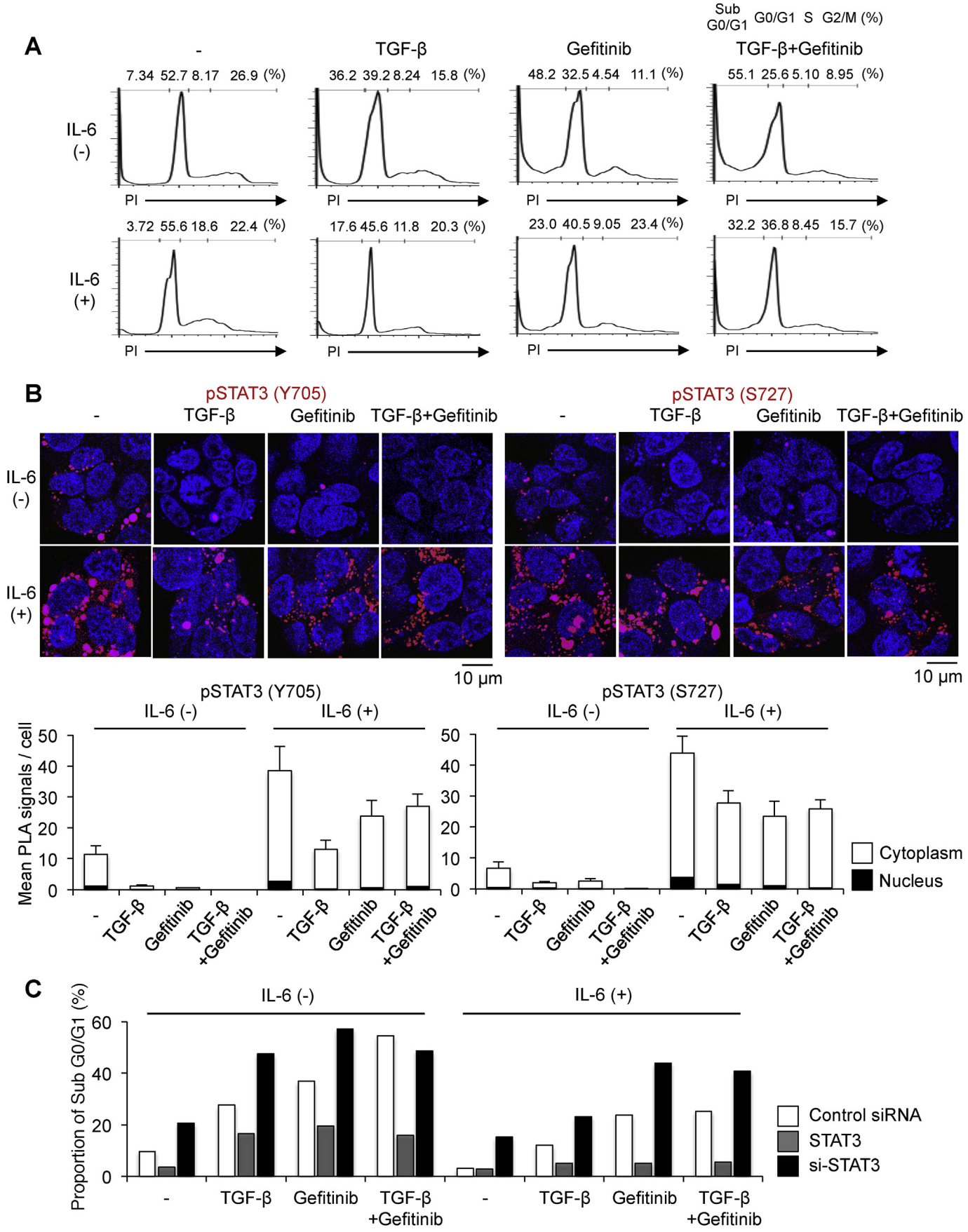

Fig. 1. IL-6 and STAT3 render HCC827 adenocarcinoma cells gefitinib-resistant. (A) HCC827 cells were treated with various combinations of gefitinib, IL-6 and TGF-β. (B) STAT3 phosphorylated at Y705 or S727 was detected by PLA. Signals were quantified using BlobFinder software (scale bars: 10 μm, nucleus: black, cytoplasm: white, $n = 10$ fields). Data are mean + s.d. (C) HCC827 cells were transfected with STAT3 cDNA, STAT3 siRNA or control siRNA and treated with various combinations of gefitinib, IL-6 and TGF-β. DNA contents were determined by PI staining using flowcytometry (A, C). Data are representative of three independent experiments.

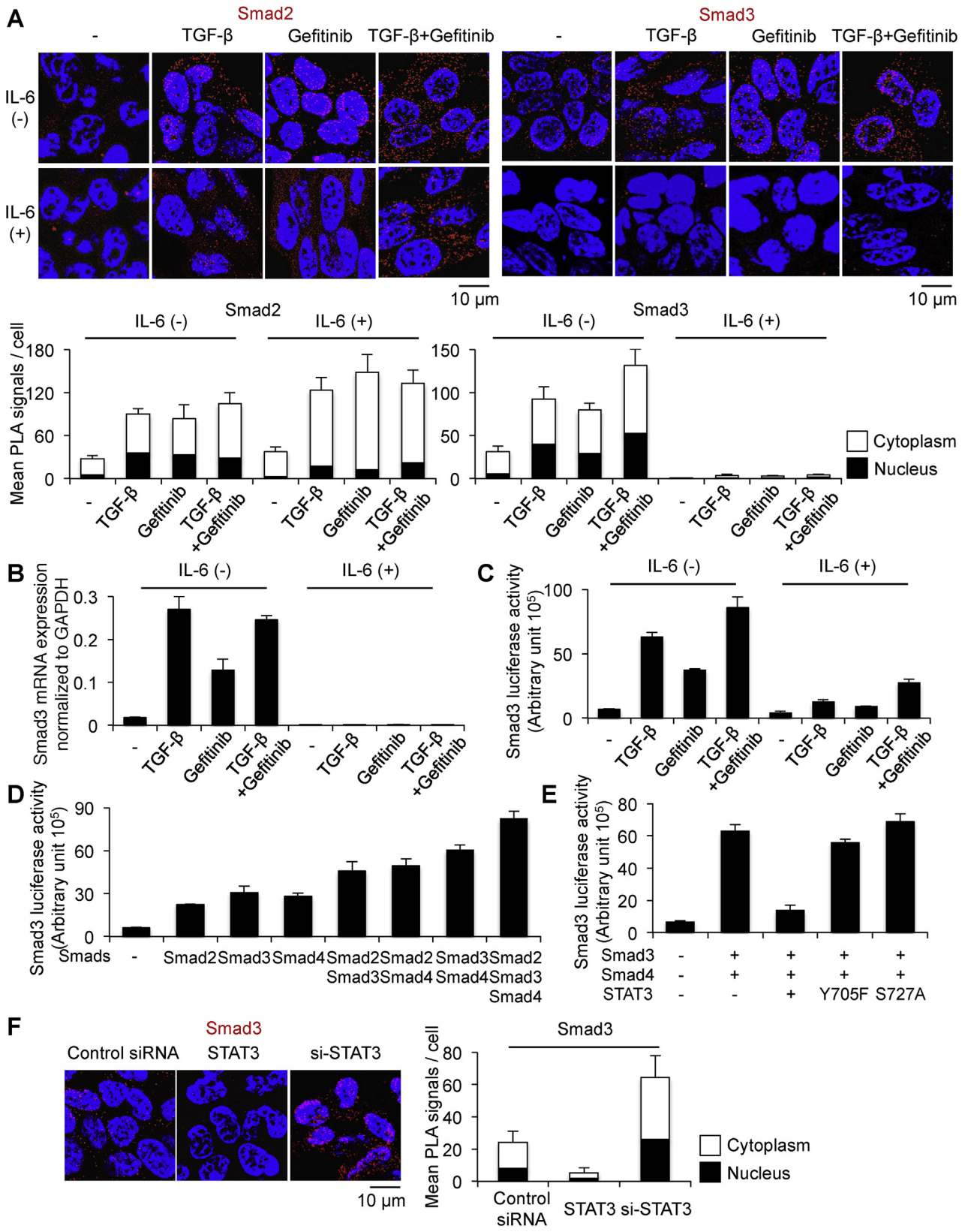

Fig. 2. STAT3-mediated IL-6 signaling represses Smad3 in HCC827 adenocarcinoma cells. HCC827 cells were treated with gefitinib and TGF-β in the presence or absence of IL-6. (A) Expression of Smad2 and Smad3 proteins in HCC827 cells was determined by PLA. (B) The expression of Smad3 mRNA relative to GAPDH in HCC827 cells was determined by quantitative RT-PCR. (C) HCC827 cells transfected with the *Smad3* promoter reporter construct were treated with gefitinib and TGF-β in the presence or absence of IL-6. (D) HCC827 cells were transfected with the *Smad3* promoter reporter construct with the indicated combinations of Smad2, Smad3 and Smad4. (E) HCC827 cells were transfected with the *Smad3* promoter reporter construct with the indicated combinations of Smad3, Smad4, STAT3, STAT3 mutants: Y705F or S727A. Firefly luciferase activity was measured with a luminometer and normalized to β-galactosidase activity. Each experiment was performed in triplicate. (F) Smad3 protein in HCC827 cells transfected with STAT3 cDNA, STAT3 siRNA or control siRNA was detected by PLA. Signals were quantified using BlobFinder software (scale bars: 10 μm, nucleus: black, cytoplasm: white, $n = 10$ fields). Data are mean + s.d. Data are representative of three independent experiments.

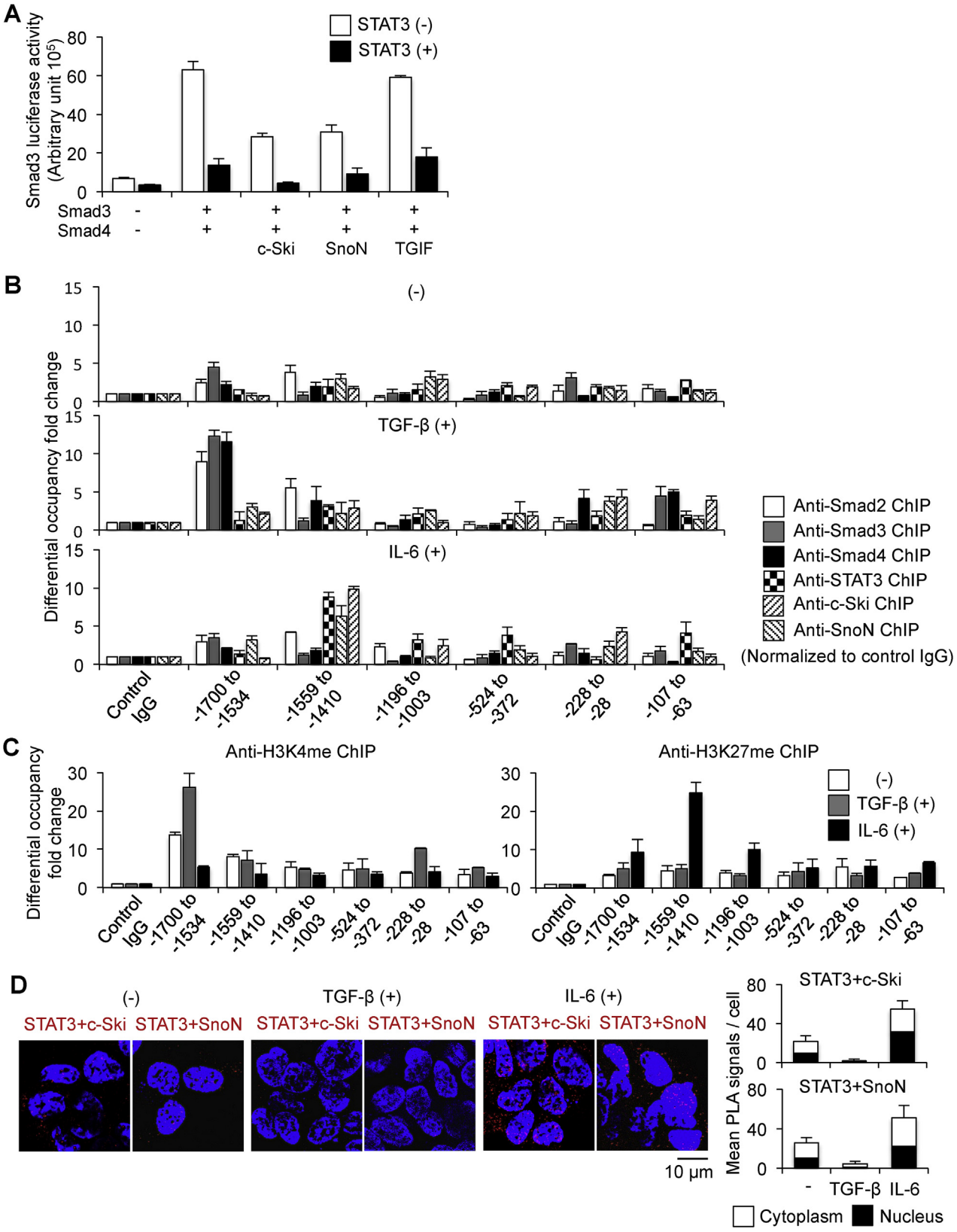

Fig. 3. Phosphorylated STAT3 represses the transcription of the *Smad3* gene in synergy with c-Ski and SnoN. (A) HCC827 cells were transfected with the *Smad3* promoter reporter construct with the indicated combinations of Smad3, Smad4, STAT3, c-Ski, SnoN and TGIF. Firefly luciferase activity was measured with a luminometer and normalized to β-galactosidase activity. Bindings of Smad2/3/4, STAT3, c-Ski and SnoN to (B) the *Smad3* proximal promoter region and (C) trimethylated histone H3 Lys4 (H3K4me3) and trimethylated histone H3 Lys 27 (H3K27me3) in HCC827 cells were determined by ChIP. Each experiment was performed in triplicate. (D) Protein interactions between STAT3 and c-Ski, STAT3 and SnoN in HCC827 cells treated with or without TGF-β or IL-6 were detected by PLA. Signals were quantified using BlobFinder software (scale bars: 10 μm, nucleus: black, cytoplasm: white, $n = 10$ fields). Data are mean + s.d. Data are representative of three independent experiments.

regulated remain largely unknown. Downregulation of either of R-Smads could be one of the mechanisms to distinguish their distinct functions [18]. Smad3 is downregulated through decreased transcription in lung epithelial cells [31] or through decreased gene activation as well as increased protein degradation in glomerular mesangial cells [32]. Here, we show that IL-6-Stat3 signaling represses transcription of Smad3, thereby inducing gefitinib/TGF-β resistance in HCC827 cells. Similar and synergistic effect of gefitinib with TGF-β on HCC827 cells might be attributed to induction of TGF-β by gefitinib [33]. Pro-inflammatory cytokine signaling pathways antagonize TGF-β through upregulation of inhibitory Smad, Smad7 [19,20]. Recent report shows that direct crosstalk between Stat3 and Smad3 antagonizes TGF-β [34]. Ski and the closely related SnoN are oncogenes, which act as transcriptional co-repressors in TGF-β signaling through interaction with Smads [24–26]. We show that c-Ski/SnoN cooperate with Stat3 to antagonize TGF-β by repressing Smad2/3/4-induced transcription of the *Smad3* gene. Future studies are required to clarify the relevance of repression of Smad3 to the interactions of c-Ski/SnoN, Stat3 and Smads in inflammation and carcinogenesis.

Stat3 activated through IL-6/JAK1 promotes cell survival and erlotinib resistance in EGFR-addicted NSCLC cell lines [23]. TGF-β also plays pivotal roles in cell survival [16–18]. Smad3 sensitizes hepatocytes to apoptosis through down-regulation of Bcl-2 [35] or

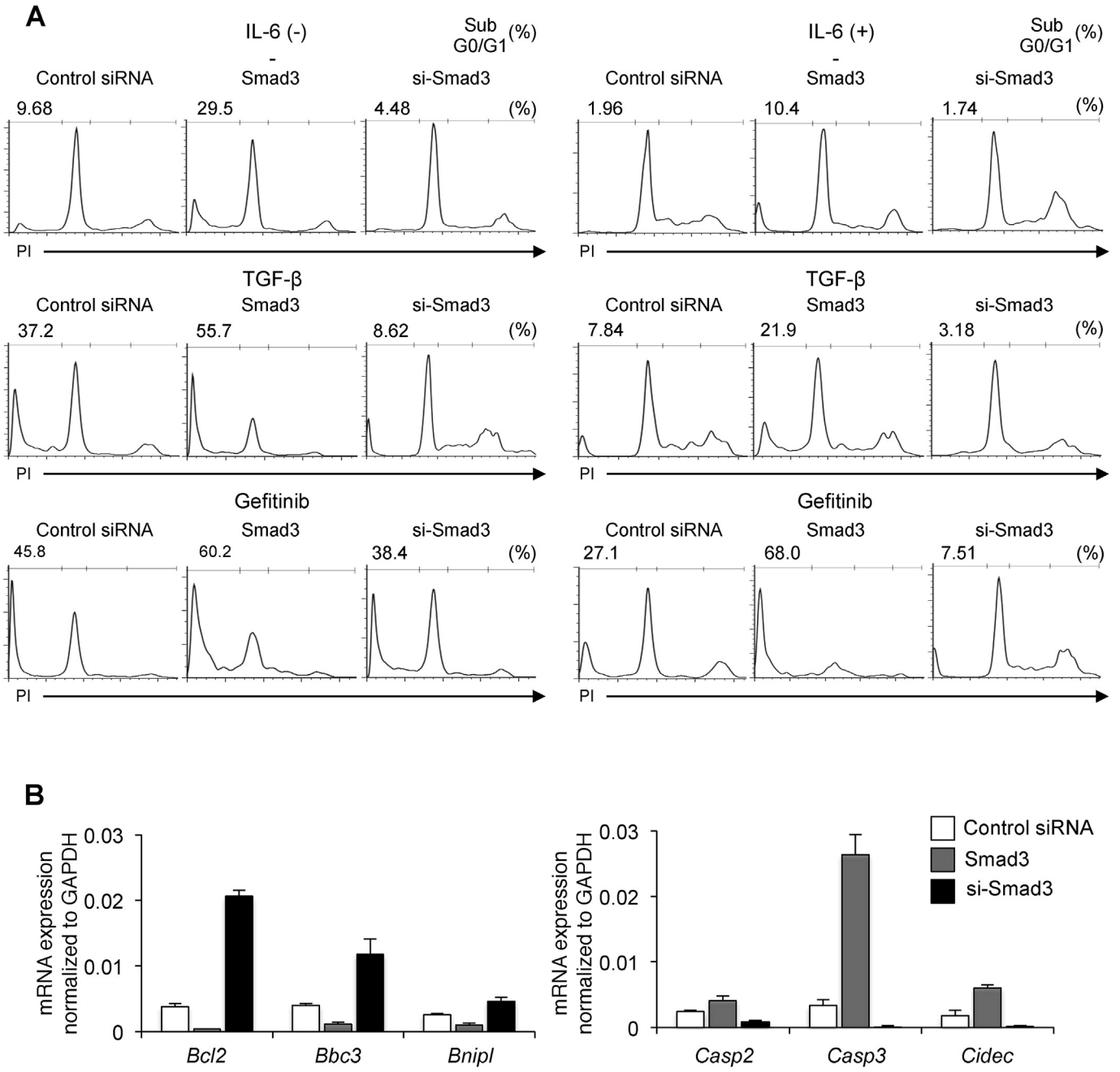

Fig. 4. Smad3 enhances gefitinib-induced apoptosis of HCC827 cells. (A) HCC827 cells were transfected with Smad3 cDNA, Smad3 siRNA or control siRNA and treated with gefitinib with or without TGF-β or IL-6. DNA contents were determined by PI staining using flowcytometry. (B) The expression of Caspase2, Caspase3, Cidec, Bcl2, Bbc3 and Bnipl mRNA relative to GAPDH in HCC827 cells transfected with Smad3 cDNA, Smad3 siRNA or control siRNA was determined by quantitative RT-PCR. Each experiment was performed in triplicate. Data are mean + s.d. Data are representative of three independent experiments.

through cleavage of Bcl-2-associated death promoter (BAD) protein [36]. TGF-β/Smad3 induces apoptosis through inducing and activating caspase-3 [37]. Our findings that Smad3 significantly prevented IL-6-induced gefitinib resistance in HCC827 cells as upregulating caspase3 and downregulating Bcl2 are consistent with these previous reports. Stat3 also targets similar set of apoptosis-related genes [38]. Involvement of crosstalk between Smads and Stat3 in regulation of apoptosis in IL-6-induced gefitinib resistance is yet to be determined.

In summary, present study elucidated the novel mechanism how IL-6/Stat3 induce gefitinib resistance in highly sensitive HCC827 cells. IL-6 enhanced phosphorylation of Stat3 at Y705 and S727, which interacted with the Ski family of proto-oncoproteins, c-Ski and SnoN to repress Smad2/3/4-induced transcription of the *Smad3* gene. Repression of Smad3 resulted in resistance to gefitinib-induced apoptosis, whereas forced expression of Smad3 restored IL-6-induced gefitinib resistance. Smad3 induced apoptosis by upregulating pro-apoptotic genes as downregulating anti-apoptotic genes. Our findings suggest that derepression of Smad3 might prevent inflammation-induced gefitinib-resistance at early stages of NSCLCs with gefitinib-sensitizing EGFR mutation while they retain sensitivity to tumor suppressor effects of TGF-β.

Declaration of interest

NI receives support from AstraZeneca. Other authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2017.01.093.

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