Introduction

Radiotherapy can be a preferential management strategy for patients with inoperable cancer types, including advanced stage of non–small cell lung cancer (NSCLC), in the absence of more effective targeted therapies (1). Nevertheless, therapeutic outcomes are not fully satisfactory due to the emergence of radioresistance that is considered to be a critical obstacle that leads to the failure of radiotherapy and consequently increases mortality in patients with NSCLC (2). So far, a large number of studies have been conducted to find a way to control radioresistance and develop potent adjuvants for radiotherapy that enhance treatment efficacy (3, 4). A profound understanding of the molecular events associated with therapeutic resistance would greatly advance the discovery of drugs enabling to modulate radiation-induced signaling responses to improve the prognosis of patients with NSCLC (5–7).

We previously found that the p21-activated Ser/Thr kinase 1 (PAK1) kinase has an essential role in tumorigenesis and cell survival in many cancers, but its regulation is not fully understood. In this study, we showed that in response to irradiation of lung cancer cells, PAK1 was upregulated, tyrosine phosphorylated, and translocated to the nucleus. Tyrosine phosphorylation relied upon JAK2 kinase activity and was essential for PAK1 protein stability and binding to Snail. This radiation-induced JAK2–PAK1–Snail signaling pathway increased epithelial–mesenchymal transition (EMT) by regulating epithelial and mesenchymal cell markers. Notably, JAK2 inhibitors mediated radiosensitization and EMT blockade in a mouse xenograft model of lung cancer. Taken together, our findings offered evidence that JAK2 phosphorylates and stabilizes functions of PAK1 that promote EMT and radioresistance in lung cancer cells, with additional implications for the use of JAK2 inhibitors as radiosensitizers in lung cancer treatment. Cancer Res; 74(19): 5520–31. ©2014 AACR.

Abstract

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by epithelial growth factor receptor (EGFR), a well-known receptor Tyr kinase (16). Altogether, these data serve as the critical evidence demonstrating that the modulation of PAK1 activity by Tyr phosphorylation may play a significant role in the control of cellular responses to various stimuli.

JAK2 is a Tyr kinase, a Janus kinase family member. JAKs are well-known to activate STAT signaling, leading to regulation of the gene expression that influences processes associated with tumor malignancy including cell-cycle progression, angiogenesis, metastasis, and immune evasion (17, 18). Many investigations have demonstrated that JAKs and STATs are highly activated in cases of lung cancer and implied that JAKs as upstream regulators can be promising targets for lung cancer therapy (19, 20). For this, small-molecule compounds targeting JAKs and their signaling activity have been currently developed as chemotherapeutic reagents. However, only a few studies have reported a relationship between JAKs and radiation-associated signaling. It has been demonstrated that the induction of c-jun expression in irradiated cells requires the activation of JAK3, but is impervious to the activation status of JAK1 and JAK2 (21). A recent study using TG101209, a small-molecule inhibitor of JAK2, found that JAK2 inhibition could be a promising strategy for lung cancer treatment (22). Therefore, it is important to define the role of the JAK2 pathway in irradiated solid tumors, because better therapies are desperately needed to treat these malignancies.

The purpose of this study was to elucidate the mechanisms and key molecules that confer radioresistance in NSCLC cells. We demonstrated that IR-dependent phosphorylation of PAK1 on Tyr residues by JAK2 and its subsequently increased stability play an important role in survival signal transduction in NSCLC cells. Our findings provide a possible explanation of how NSCLC cells can acquire and regulate resistance to radiation. Furthermore, we suggest that JAK2 and PAK1 could be attractive pharmaceutical targets for overcoming radioresistance of NSCLC and ultimately contribute to the effective radiation treatment of lung cancer.

Materials and Methods

Chemicals, antibodies, and reagents

Chemicals, antibodies, and reagents used are described in Supplementary Materials and Methods.

Cell lines, cell culture, irradiation, and drug treatment

A549 and NCI-H460 cells were acquired from the http://www.atcc.org/ American Type Culture Collection (ATCC), authenticated, and maintained in early passages, no more than 6 months after receipt from the ATCC. Cells were grown in RPMI-1640 medium supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C in 95% air/5% CO2. The cells were exposed to a single dose of γ-rays using a Gamma Cell-40 Exactor (Nordion International, Inc.) at a dose rate of 0.81 Gy/min. Flasks containing the control cells were placed in the irradiation chamber but not exposed to radiation. The cells were treated with the indicated drug dissolved in dimethyl sulfoxide (DMSO) for 4 hours.

Northern blot analysis

Northern blot analysis was performed as described previously (23). The detailed conditions and primers used for mRNA expression are listed in Supplementary Materials and Methods.

Western blot analysis, immunoprecipitation, and in vivo kinase assay

Following the experimental treatment, Western blot analysis, immunoprecipitation (IP), and in vivo kinase assay studies were performed as previously described (4, 6, 23). Detailed procedures are described in Supplementary Materials and Methods.

Transient transfection

For transient transfection, cells were plated at a density of 5 × 105 cells in 6-well dishes and incubated for 4 hours. The cells were transiently transfected with the indicated plasmid using Lipofectin (Invitrogen) or the siRNA oligonucleotides (10 nmol/L) targeting EGFR, c-Abl, JAK1, JAK2, and JAK3 using DharmaFECT 1 (Dharmacon) according to the manufacturer’s instructions.

Interactome analysis

For interactome analysis, cells were transfected with Myc-PAK1 and harvested after being irradiated (4 Gy, 2 hours). Nuclear extract was then used for double immunoaffinity purification of PAK1-containing complexes as previously described (24). Detailed procedures are summarized in Supplementary Materials and Methods.

Luciferase reporter gene assay

Following cotransfection with E-cadherin (E-cad-Luc) or occludin Luciferase reporter gene (Occlu-Luc) and specific combination of plasmids as indicated, the medium was changed and the cells were treated with siRNA, drug, and/or irradiation. After 1 hour, the cells were washed twice with cold PBS and lysed in reporter lysis buffer (Promega). After vortexing the lysates and centrifugation at 12,000 × g for 1 minute at 4°C, the cell extract and luciferase assay reagent (Promega) were mixed at room temperature, and placed in a luminometer (AutoLumat LB 953, EG & G Berthold) to measure luciferase activity.

Cell assay (3D culture) and immunofluorescence staining

The three-dimensional (3D) acini culture was conducted to detect morphologic changes of the epithelial acini as previously described (25). Detailed conditions are described in Supplementary Materials and Methods.

Transwell cell migration assay

A cell migration assay was performed using a 24-well Transwell chamber (Corning). Cells (1 × 105 in serum-free RPMI-1640 medium) cultured with or without specific treatments (transfection, IR, and/or drugs) for 60 (A549) or 72 hours (NCI-H460) were seeded in the upper chamber of a 5 μm pore-size insert. The lower chamber was filled with RPMI-1640 medium containing 2% FBS. After 6 hours, the upper
membrane surface was wiped with a cotton swab to remove cells that had not migrated into the lower chamber. Migrated cells attached to the lower membrane surface were fixed with 4% paraformaldehyde, stained with hematoxylin, and counted. The migration index was calculated and normalized to the number of untreated cells that had migrated and the results were based on the relative number of cells in a randomly selected field from three representative experiments.

**Wound-healing assay**

Cell monolayers were scratched using a 200-μL pipette tip after the cells had reached 70% confluence in RPMI-1640 medium supplemented with 1% FBS. The cells were further incubated with fresh medium with or without treatment for 18 and 30 (A549) or 24 and 48 hours (NCI-H460). Photomicrographs were then taken at ×100 magnification with an Olympus IX71 fluorescence microscope (Olympus Optical Co. Ltd.).

**Real-time qRT-PCR**

The level of epithelial–mesenchymal transition (EMT)–related gene expression was measured using qRT-PCR as previously described (23). The detailed conditions and primers used for mRNA expression are listed in Supplementary Materials and Methods including Supplementary Table S1.

**Tumor xenografts in nude mice**

Six-week-old male BALB/c athymic nude mice (Central Lab Animals Inc.) were injected with 2 × 10⁶ A549 or NCI-H460 cells into the flank, and tumors were allowed to develop. Upon identification of a palpable tumor (minimal volume of 200 mm³), DMSO or drug (200 μg/kg body weight) was administered i.p. every day for 25 days. The animals were also irradiated with 10 Gy once a week for 3 weeks. Tumor length (L) and width (W) were measured with a caliper and tumor volumes were calculated with the formula (L × W²)/2. At the end of the treatment period, the animals were euthanized and the tumors were used for biochemical studies. Animal care protocol is detailed in Supplementary Materials and Methods.

**Statistical analysis**

All numeric data are presented as the mean ± SD or SEM from at least three independent experiments. The results were analyzed using a one-way ANOVA for ranked data followed by Tukey honestly significant difference test, and the two-way ANOVA for ranked data followed by a Bonferroni posttest. Prism 4 software (GraphPad Software) was used to conduct all statistical analyses. A P value of <0.05 was considered to be statistically significant.

**Results**

**IR induces overexpression, Tyr phosphorylation, and nuclear localization of PAK1 in NSCLC cells**

Nuclear and cytoplasmic PAK1 is strongly expressed in squamous NSCLC cells (13). On the basis of this information and our previous transcriptome analysis (8), we measured the expression of PAK1 in irradiated A549 and NCI-H460 cells in the present study. As shown in Fig. 1A and B, IR exposure of A549 and NCI-H460 cells dramatically induced both mRNA and protein expression of PAK1 in a dose-dependent manner. Next, IR-dependent molecular modification of PAK1 was investigated because PAK1 was known to be phosphorylated for its activity. In both NSCLC cells, IR-induced PAK1 phosphorylation on Tyr sites was observed, whereas phosphorylation at Ser/Thr sites was not detected (Fig. 1C and Supplementary Fig. S1A). On the basis of the literature and 3D structure of PAK1 (PDB ID: 1F3M), to further determine which residues are the targets of Tyr kinase in PAK1, we used each mutant (Y153F, Y201F, Y295F, or Y3F) as a substrate for an in vivo kinase assay (14, 26). Tyr phosphorylation was reduced in Y153F, Y201F, or Y285F mutants of PAK1, and completely eliminated from the PAK1 Y3F (Y153F/Y201F/Y285F) mutant (Fig. 1D and Supplementary Fig. S1B). These findings indicated that Y153, Y201, and Y285 of PAK1 are required for Tyr phosphorylation in irradiated A549 and NCI-H460 cells. It has been reported that PAK1 contains nuclear localizing signal sequences and signal-dependent nuclear localization is required for transcriptional modulation of PAK1 (27, 28). To determine whether IR can change the subcellular localization of PAK1, the protein levels of PAK1 in the cytoplasmic or nuclear region were measured in irradiated A549 and NCI-H460 cells. As shown in Fig. 1E, IR increased the amount of nuclear PAK1 in a time-dependent manner. To further examine whether IR-induced Tyr phosphorylation affects the nuclear localization of PAK1, we conducted an immunoprecipitation assay of the cytoplasmic or nuclear fraction from irradiated A549 and NCI-H460 cells after transfection with HA-PAK1 [wild-type (WT) or Y3F]. We found that IR-induced nuclear localization of the PAK1 Y3F mutant was significantly inhibited compared with that of PAK1 WT (Fig. 1F). Collectively, these data suggest that IR induces overexpression, Tyr phosphorylation, and, subsequently, nuclear localization of PAK1.

**JAK2 phosphorylates PAK1 in irradiated NSCLC cells**

Because we found that IR induced the phosphorylation of PAK1 on Tyr residues in NSCLC cells, we further examined upstream kinases of PAK1. siRNA specific for each Tyk kinase (EGFR, c-Abl, JAK1, JAK2, and JAK3) was prepared and an in vivo kinase assay was performed on the cells expressing these siRNA molecules (Fig. 2A, inset). Inhibition of JAK2 expression led to complete abolishment of PAK1 phosphorylation in irradiated A549 and NCI-H460 cells; JAK2 was therefore selected as a candidate for upstream kinase of PAK1 (Fig. 2A, inset). We next assessed the interaction between JAK2 and PAK1 in both irradiated NSCLC cells. It was observed that JAK2 was activated by irradiation and interacted with PAK1 in cytoplasm (Fig. 2B and Supplementary Fig. S2). Moreover, PAK1 was phosphorylated at Tyr sites by JAK2 WT but not by JAK2 kinase-dead (KD) after IR exposure (Fig. 2C). To further investigate whether the Y153, Y201, and Y285 phosphorylation sites of PAK1 are targets of JAK2, an in vivo kinase assay was performed with the PAK1 Y3F mutant. As shown in Fig. 2D, JAK2-induced PAK1 phosphorylation was eliminated by the Y3F mutation in PAK1, indicating that the suggested three residues of PAK1 are required for JAK2-mediated phosphorylation in irradiated A549 and NCI-H460 cells. Direct
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Figure 1. IR induces overexpression, Tyr phosphorylation, and nuclear localization of PAK1 in NSCLC cells. A, IR-induced PAK1 mRNA expression was detected by Northern blot analysis. After irradiation (0, 2, 4, or 6 Gy), the cells were harvested and total RNA was extracted. Total RNA was analyzed using an [c-32P]-labeled PAK1 cDNA probe. GAPDH was used for normalization. B, IR-induced PAK1 protein expression was detected by Western blot analysis. C, IR-induced PAK1 phosphorylation was verified by an in vivo kinase assay. After irradiation (4 Gy) for 0, 1, 2, or 3 hours, the cells were harvested and cell lysates were subjected to an immunoprecipitation (IP) assay with an anti-PAK1 antibody followed by Western blot analysis for pSer/Thr and pTyr. D, IR-induced PAK1 phosphorylation at three Tyr residues (Y153, Y201, and Y285) was confirmed by an in vivo kinase assay using PAK1 Y153F, Y201F, Y285F, and Y3F mutants (WCL, whole cell lysates). Cells were harvested after irradiation (4 Gy) for 2 hours and cell lysates were immunoprecipitated with an anti-HA antibody and analyzed by Western blot analysis for pTyr. E, IR-induced translocation of PAK1 from the cytoplasm into the nucleus was assayed by Western blot analysis after cytoplasmic or nuclear fractionation (CE, cytoplasmic extract; NE, nuclear extract). Tubulin and Lamin A/C were used as markers for the CE and NE, respectively. F, involvement of IR-activated nuclear localization and phosphorylation of PAK1 was determined by an immunoprecipitation assay and Western blot analysis. After irradiation, the cells transiently transfected with PAK1 (WT or Y3F mutant) were harvested to conduct cytoplasmic or nuclear fractionation. Each extract was immunoprecipitated with an anti-HA antibody and detected by Western blot analysis using an anti-pTyr antibody.

phosphorylation of PAK1 by JAK2 was confirmed by treating A549 and NCI-H460 cells with two JAK2-specific inhibitors, TG101209 and TG101348. IR-induced PAK1 phosphorylation by JAK2 was significantly diminished following treatment with TG101209 and TG101348, but not AG1478 (an EGFR inhibitor) or STI-571 (a c-Abl inhibitor; Fig. 2E). Therefore, we propose that IR-activated PAK1 phosphorylation at Tyr residues is directly mediated by JAK2 in NSCLC cells.

**Tyr phosphorylation is required for maintaining PAK1 protein stability in irradiated NSCLC cells**

To explore the biologic effects of PAK1 Tyr phosphorylation, both HA-PAK1 WT and HA-PAK1 Y3F were expressed in NSCLC cells by transient transfection. As shown in Fig. 3A, HA-PAK1 Y3F was expressed at a lower level under IR compared with the WT construct. Treatment with proteasome inhibitors restored the mutant protein level, indicating that Tyr phosphorylation of PAK1 modulates protein stability. Indeed, the estimated half-life of PAK1 WT (12 hours) was significantly longer than that of the Y3F mutant (4 hours; Fig. 3B). These results indicate that cellular PAK1 is subject to proteasome-dependent degradation and that phosphorylation on Tyr residues prevents PAK1 degradation. In line with these findings, proteasome inhibitors caused a more pronounced accumulation of poly-ubiquitinated PAK1 Y3F than PAK1 WT (Fig. 3C).

Previously, we observed that PAK1 is phosphorylated by JAK2 after irradiation (Fig. 2). To test whether the same mechanism also mediates the poly-ubiquitination that is associated with PAK1 Y3F, JAK2 activation was blocked by a JAK2-specific inhibitor. The results showed that JAK2 inhibition did increase the level of poly-ubiquitinated PAK1 WT, indicating that the protein stability of PAK1 is controlled by a signaling pathway that is dependent on Tyr phosphorylation of PAK1 induced by JAK2 after irradiation in NSCLC cells (Fig. 3D). According to these data, we propose that IR-activated PAK1 Tyr phosphorylation by JAK2 is directly related to PAK1 protein stabilization in NSCLC cells.

**PAK1 interacts with Snail in irradiated NSCLC cells**

Because we found that PAK1 was phosphorylated by IR-activated JAK2 and localized to the nucleus, we further set out to identify a PAK1-interacting proteome in irradiated NSCLC cells to elucidate the molecular functions of PAK1 in the nucleus. The strategy used to identify nuclear proteins associated with nuclear PAK1 is presented in Fig. 4A. Nuclear extracts were prepared from A549 cells cultured with IR and soluble nuclear protein complexes were separated to reduce sample complexity. Complexes containing PAK1 were purified using double immunoaffinity purification, and the identity of PAK1 interactors was determined by repeated rounds of

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The phosphorylation of Snail on S246 by PAK1 is essential for phosphorylation-dependent interaction with Snail (Fig. 4C and D). Unlike PAK1 WT, PAK1 Y3F interacted with Snail, suggesting that the interaction of PAK1 with Snail in irradiated NSCLC cells is phosphorylation-dependent. A previous report has shown that the phosphorylation of Snail on S246 by PAK1 is essential for Snail function by promoting transcriptional repression of several genes, including epithelial marker proteins (E-cadherin), in breast cancer cell lines (30). As shown in Fig. 4E, PAK1-mediated Snail phosphorylation was detected and eliminated by an S246A mutation in Snail, indicating that S246 of Snail is required for PAK1-mediated phosphorylation in irradiated NSCLC cells. To explore a potential effect of PAK1 binding of IR-activated PAK1 with JAK2 was measured with an immunoprecipitation assay. After irradiation for 2 hours, cells transfected with JAK2 WT or KD were subjected to Western blot analysis using an anti-PAK1 antibody. D, JAK2-mediated PAK1 phosphorylation at Y153, Y201, and Y285 was confirmed by an in vivo kinase assay with a PAK1 Y3F mutant. Cells were harvested after irradiation (4 Gy) for 2 hours. Immunoprecipitant of HA (PAK1) was subjected to Western blot analysis using an anti-pTyr antibody. E, JAK2-mediated PAK1 phosphorylation was reconfirmed using JAK2-specific inhibitors (TG101209 or TG101348), an EGFR inhibitor (AG1478), and a c-Abl inhibitor (STI-571). After treatment of each inhibitor and irradiation for 2 hours, cells were harvested and were immunoprecipitated with an anti-PAK1 antibody followed by Western blot analysis using an anti-pTyr antibody.
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(Fig. 4F). We next showed that inhibition of the PAK1 pathway by PAK1-specific siRNA resulted in increased transcriptional activity of the E-cadherin promoter in irradiated NSCLC cells. The involvement of phosphorylated PAK1 in the ability of Snail to repress E-cadherin promoter activity was confirmed by Western blot analysis. Ectopic expression of PAK1 WT or the Y3F mutant in NSCLC cells with or without IR (4 Gy; 6 hours incubation) and proteasome inhibitors (a mix of 5 μmol/L MG132 and 5 μmol/L MG262, 24 hours incubation). B, the half-life of IR-activated PAK1 WT or Y3F mutant with cycloheximide (CHX; 50 μmol/L) in the presence of proteasome inhibitors was estimated by Western blot analysis. The level of HA-PAK1 WT or Y3F mutant expression at time point 0 (lane 3) was set at 100%. C, the accumulation of poly-ubiquitinated IR-activated PAK1 Y3F mutant was measured. NSCLC cells expressing PAK1 WT or Y3F mutant were treated with or without proteasome inhibitors as described in A. HA-PAK1 WT or Y3F-mutant protein was then immunoprecipitated with an anti-HA antibody and Western blot analysis was performed with an anti-ubiquitin antibody. D, the direct relationship between JAK2-dependent phosphorylation of PAK1 and poly-ubiquitination with irradiation in NSCLC was confirmed by the same experiment described in C with a JAK2-specific inhibitor, TG101209.

Inhibition of Tyr phosphorylation of PAK1 reduces IR-dependent EMT in NSCLC cells

Several lines of evidence have demonstrated the critical role of PAK1 and Snail in the EMT (30, 32). To explore a potential role for PAK1 phosphorylation in Snail-mediated EMT and the effect of a JAK2 inhibitor as an EMT suppressor, we monitored morphologic changes of NSCLC cells. A549 and NCI-H460 cells were grown on a thick layer of Matrigel to form epithelial acini (3D culture model). This system has been used extensively to identify morphologic changes that can perturb the normal cancer growth architecture (33, 34). In this 3D culture model, irradiated NSCLC cells were distinguishable from the control cells and formed an increased number of acini that invaded the Matrigel-containing 3D matrix. Interestingly, the observed morphologic changes of the epithelial acini were abrogated by the addition of TG101209, indicating that the inhibition of PAK1 Tyr phosphorylation reduced IR-dependent EMT in the NSCLC cells (Fig. 5A). Next, we measured the migration capacity and EMT marker expression of NSCLC cells treated with TG101209. NSCLC cells treated with the JAK2 inhibitor exhibited reduced motility as determined by both Transwell cell migration and wound-healing assays (Fig. 5B and C). Rhamnetin and cirsiliol were administered as positive controls of EMT inhibitors (35).

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**PAK1 interacts with Snail in irradiated NSCLC cells.** A, schematic presentation of the strategy used to identify the PAK1 nuclear interactome in A549 cells. B, purified PAK1 complexes in nonirradiated or irradiated A549 cells were separated by PAGE and visualized by silver staining. The silver-stained gel was analyzed by mass spectrometry (peptide mass fingerprint, PMF). It was shown that three bands were especially increased by irradiation. The bands indicated by an arrowhead (a, b, and c) correspond to Snail, EF1a, and FKHR, respectively. C, in vivo binding of IR-activated PAK1 with Snail in the nucleus of NSCLC cells was confirmed by an immunoprecipitation (IP) assay. After irradiation, the cells transiently transfected with PAK1 WT or Y3F mutant were harvested to conduct cytoplasmic or nuclear fractionation. Each extract was subjected to an immunoprecipitation assay with an anti-PAK1 antibody followed by Western blot analysis for Snail. D, interaction of endogenous PAK1 with endogenous Snail was measured by an immunoprecipitation assay. After irradiation, the cells were harvested to conduct cytoplasmic or nuclear fractionation. Each extract was subjected to an immunoprecipitation assay with an anti-PAK1 antibody followed by Western blot analysis for Snail. E, interaction of endogenous PAK1 with endogenous Snail was measured by an immunoprecipitation assay. After irradiation, the cells were harvested to conduct cytoplasmic or nuclear fractionation. Each extract was subjected to an immunoprecipitation assay with an anti-FLAG antibody (Snail) followed by Western blot analysis using an anti-pSer/Thr antibody. F, effect of PAK1 on the ability of Snail to repress E-cadherin (or occludin) promoter activity was assessed with a luciferase assay. Error bars, ± SD (n = 3); *, P < 0.05 compared with E-cad (or Occlu)-Luc plasmid-transfected nonirradiated cells; **, P < 0.05 compared with E-cad (or Occlu)-Luc plasmid-transfected irradiated cells (two-way ANOVA, Bonferroni posttest).

(Fig. 5D and E). Reduced EMT was also observed in the NSCLC cells in which PAK1 expression was knocked down. Taken together, these results suggest that the inhibition of PAK1 activation in NSCLC cells suppresses the EMT.

**JAK2 inhibitor increases in vivo radiosensitization and decreases in vivo EMT in a xenograft mouse model**

In our wound-healing assay, we observed that TG101209 treatment prevented not only the EMT but also the proliferation of IR-activated NSCLC cells (Fig. 5C). To evaluate the combined effects of JAK2 inhibition with IR on tumor growth in vivo, a xenograft mouse model was established (Fig. 6A). In vivo data from nude mice bearing tumors formed by A549 and NCI-H460 cells indicated that TG101209 had an in vivo radiosensitization effect (Fig. 6B). Tumor volumes of the mice treated with IR and TG101209 were significantly reduced by approximately 57% (the group with tumors formed by A549 cells) and 50% (the group injected with NCI-H460 cells) on day 25 compared with mice receiving radiation alone. Moreover, IR-induced expression of PAK1 and EMT-related proteins was significantly reduced in the extracted tumor tissue lysates when TG101209 was administered in the mice (Fig. 6C). Thus, we suggest that the JAK2 inhibitor significantly increased in vivo radiosensitization while inhibiting the EMT.
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Figure 5. Inhibition of PAK1 Tyr phosphorylation reduces IR-dependent EMT in NSCLC cells. A, morphologic changes of NSCLC cells treated with a JAK2 inhibitor were monitored with a 3D culture model. NSCLC cells treated with or without IR and TG101209 were grown in 3D cultures and fixed after 20 hours (A549) or 28 hours (NCI-H460). The cells were permeabilized and stained for tubulin (green) and with DAPI (blue). B, the inhibitory effects of a JAK2 inhibitor were monitored with a 3D culture model. NSCLC cells treated with or without IR and TG101209 were grown in 3D cultures and fixed after 20 hours (A549) or 28 hours (NCI-H460). The cells were permeabilized and stained for tubulin (green) and with DAPI (blue). C, effects of a JAK2 inhibitor on the protein expression of E-cadherin, vimentin, and fibronectin in irradiated NSCLC cells were analyzed by Western blot analysis. Error bars, ± SD (n = 3); **P < 0.01 compared with nonirradiated cells, ***P < 0.001 compared with irradiated cells (one-way ANOVA, Tukey honestly significant difference test, or two-way ANOVA, Bonferroni posttest).

Discussion

Many researchers have focused on developing potent radiosensitizers to overcome radioresistance and to increase the therapeutic efficacy in patients with NSCLC. Given the inherent insensitivity to cytotoxic agents, identifying molecules that drive the growth, survival, and metastasis of lung cancer cells is critical for the development of novel therapeutic reagents. In the same context with identification of the biomarkers, we found that JAK2 and PAK1 are promising pharmacologic targets for the radiosensitization of NSCLC cells because Tyr phosphorylation of PAK1 by JAK2 was shown to prevent cell death and to promote cell invasion. In the present study, PAK1 mRNA and protein expression was upregulated, and the protein localized to the nucleus in irradiated NSCLC cells. In...
In addition to nuclear localization, the stability of PAK1 protein increased through IR-dependent Tyr phosphorylation of PAK1 by JAK2. We also found that activation of the JAK2–PAK1–Snail pathway by IR was important for radioresistance primarily via EMT induction (Fig. 7). On the basis of these findings, we suggest that JAK2 can be a potent target as an upstream factor for Tyr phosphorylation of PAK1 associated with radiosensitive signaling in NSCLC cells.

PAK1 has an autoregulatory region (including a p21-binding domain, dimerization segment, and inhibitory switch domain) and several motifs for interaction with adaptor proteins (Nck, Grb2, and PIX) at the N-terminus, and a catalytic domain at the C-terminus (26). PAK1 activity is known to be inhibited by phosphorylation of Ser/Thr residues of substrates (40). On the basis of the location of Y285 at the ATP binding site, we expect that phosphorylation of this residue might help stabilize the inhibitory conformation similar to phosphorylation at S199/S203. In addition, maintenance of the PAK1 active conformation could be achieved, at least partially, by phosphorylation at Y153 because this residue also resides nearby the autophosphorylation sites, S144 and S149 (especially S144 is responsible for maximal kinase activity of PAK1; ref. 39). Phosphorylation at Y285 could also affect kinase activity because this residue is present in the N-lobe of the C-terminal catalytic domain. A previous structural study revealed that PAK1 could form an active conformation through an interaction between K299 at the N-lobe, E315 at the αC-helix, and ATP, leading to exposure to a substrate binding site and phosphorylation of Ser/Thr residues of substrates (40). On the basis of the location of Y285 at the ATP binding site, we expect that phosphorylation of this residue might help stabilize the interaction between K299 and an ATP molecule. Although it should be confirmed by further structural studies, phosphorylation of PAK1 at three Tyr residues might directly or indirectly help PAK1 to form and maintain its catalytically active conformation, resulting in the phosphorylation of downstream substrates in response to irradiation.

We observed that Tyr phosphorylation of PAK1 was mediated by JAK2, a Tyr kinase (Fig. 2). Most substrates bind to Tyr kinases through their SH2 domains. According to previous structural studies, PAK1 does not contain an SH2 domain but has a proline-rich motif for binding to SH3 domains (26, 36, 37). This structural information implies that PAK1 could be phosphorylated on Tyr residues by JAK2 through interaction with...
SH2/SH3-containing adaptor proteins that provide a bridge for facilitating spatial proximity between JAK2 primarily in near membrane and PAK1 in the cytoplasm. PAK1 has several known adaptor proteins including Nck, Grb2, and PIX (41, 42). These proteins enable membrane localization of PAK1 to be activated by other kinases including JAK2, Cdc42, and Rac1 (41, 43). On the basis of previous studies, we believed that Nck might be involved in JAK2-mediated PAK1 Tyr phosphorylation in response to irradiation (28, 41). Nck shuttles PAK1 from the cytoplasm to the plasma membrane. After membrane localization, PAK1 would be susceptible to binding to JAK2 and, subsequently activated PAK1 would dissociate from Nck. PAK1 activated by JAK2 could be therefore released from the plasma membrane into the cytoplasm and even translocate into the nucleus. In this study, we observed that activated PAK1 affected Snail-dependent cell migration in the nucleus (Fig. 4 and 5).

In addition to activating PAK1, we showed that Tyr phosphorylation increased PAK1 stability as well (Fig. 3). To understand how PAK1 stabilization is mediated by Tyr phosphorylation, it was important to determine which E3 ligase is responsible for PAK1 degradation. Although a PAK1-specific E3 ligase has not been identified, Cdc42 homologous protein (Chp) is a Rho-family GTPase that mediates ubiquitination-dependent degradation of PAK1 (44). A previous report indicated that kinase activity of PAK1 is critical for PAK1 degradation (44). According to this study, Chp could mediate PAK1 degradation only when PAK1 was activated by autophosphorylation on several Ser/Thr residues including S57, S144, S149, S199/S203, and T423. Alanine substitutions at these sites led to increased PAK1 stability. Thus, we believe that Y201 phosphorylation of PAK1 might have structurally similar property of phosphorylated moiety as phosphorylation of S199/S203, expecting PAK1 destabilization. However, PAK1 was stabilized after being Tyr-phosphorylated as shown in Fig. 3. One possible explanation is that Chp might not recognize Tyr-phosphorylated PAK1 due to different autophosphorylation patterns. Thus, JAK2-induced Tyr-phosphorylation, especially at Y201, increased PAK1 activity and stability instead of promoting PAK1 degradation. Our data are supported by findings from other investigations demonstrating that protein degradation could be inhibited by phosphorylation at Tyr residues (45, 46). For example, ubiquitination and degradation were more rapid for a mutant form of c-Kit (Y823F) than in c-Kit WT (45). In this case, mutant c-Kit-mediated downstream pathways involving Akt and Erk were negatively affected, leading to the suppression of cell survival and proliferation. However, the Y823F mutation has no effect on kinase activity of c-Kit. Another study showed that proliferating cell nuclear antigen (PCNA) was stabilized by phosphorylation on Y211, leading to increased functional activity on DNA replication (46). We showed that PAK1 without Tyr phosphorylation is susceptible to proteasome-dependent degradation in Fig. 3. Similar to c-Kit and PCNA, phosphorylation on Tyr residues is responsible for maintaining PAK1 stability and activity, leading to cell proliferation and invasion induced by Snail phosphorylation.

Several investigations have suggested that Tyr-phosphorylated PAK1 could be crucially involved in the regulation of cancer cell migration (14, 28, 47). On the basis of the mechanisms underlying EMT regulation, we focused on a nuclear factor as a downstream target of PAK1 that could confer radiosensitnt properties on NSCLC cells. We found that Snail interacted with PAK1 in the nucleus according to our interactome analysis in Fig. 4. Although this interaction was observed in the cytoplasm as well, it is unclear whether PAK1 and Snail translocated into the nucleus together or individually. Nevertheless, the interaction between PAK1 and Snail could be responsible for EMT induction through the...
suppression of E-cadherin expression in irradiated NSCLC cells. This finding could be supported by a previous study demonstrating that Snail phosphorylation at S246 by PAK1 led to nuclear accumulation of Snail and promotes cell invasiveness (30). In addition, constitutively active PAK1 (T423E mutant) enhanced Snail activation compared with PAK1 WT. T423 of PAK1 is located in the catalytic domain necessary for kinase activity. As we mentioned above, phosphorylation of PAK1 on three Tyr residues might have similar effects on kinase activity due to exposure of the catalytic domain through phosphorylation of residues in the inhibitory switch domain. In this study, even when phosphorylation at Ser/Thr residues (such as T423) of PAK1 was not detected, PAK1 could interact with Snail in response to irradiation, consequently leading to increasing cell motility through reduced E-cadherin expression.

In addition to regulating cell motility, Snail also promotes cell survival and proliferation. Snail regulates the expression of several genes mainly involved in cell invasion and migration including matrix metalloproteinase (MMP) family members (MMP1, MMP2, and MMP7) and cell–cell or cell–matrix interacting proteins (E-cadherin, integrin-66, and tissue inhibitor of metalloproteinase 3; ref. 48). Furthermore, this factor is responsible for suppressing the expression of other genes including caspase-8 and tumor-suppressor CYLD (49). It has been reported that CYLD could be repressed in a Snail-dependent manner, resulting in increased expression of cyclin D1 and N-cadherin, thus enhancing the proliferation and migration of melanoma cells (49). These findings could explain, at least in part, the hyper-growth of cells (especially NCI-H460 cells at 48 hours) treated with irradiation alone compared with that of cells treated with irradiation and each drug observed in the present study (Fig. 5C). Taken together, our findings and data from the literature indicate that PAK1-activated Snail might directly or indirectly confer radioresistance on NSCLC cells by inducing EMT and cell proliferation.

The exact molecular mechanism underlying radioresistance in NSCLC has been still largely elusive. In the present study, we demonstrated that Tyr phosphorylation of PAK1 is critical for conferring radioresistance. We have provided the first evidence of a novel regulatory mechanism of radioresistance including the functional involvement of JAK2, PAK1, and Snail in irradiated NSCLC cells (Fig. 7). We also propose that JAK2 inhibitors might be potential radiosensitizers that suppress Tyr phosphorylation of PAK1. In this study, our results demonstrated that regulation of Tyr phosphorylation of PAK1 with pharmacologic agents in combination with radiotherapy could overcome radioresistance and eventually enhance the efficiency of radiotherapy for treating NSCLC.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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PAK1 Tyrosine Phosphorylation Is Required to Induce Epithelial–Mesenchymal Transition and Radioresistance in Lung Cancer Cells

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