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mTOR Inhibitors Radiosensitize PTEN-Deficient Non-Small-Cell Lung Cancer Cells Harboring an *EGFR* Activating Mutation by Inducing Autophagy

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ABSTRACT

Clinical resistance to gefitinib, an epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor (TKI), in patients with lung cancer has been linked to acquisition of the T790M resistance mutation in activated *EGFR* or amplification of *MET*. Phosphatase and tensin homolog (*PTEN*) loss has been recently reported as a gefitinib resistance mechanism in lung cancer. The aim of this study was to evaluate the efficacy of radiotherapy in non-small-cell lung cancer (NSCLC) with acquired gefitinib resistance caused by PTEN deficiency to suggest radiotherapy as an alternative to EGFR TKIs. PTEN deficient-mediated gefitinib resistance was generated in HCC827 cells, an EGFR TKI sensitive NSCLC cell line, by PTEN knockdown with a lentiviral vector expressing short hairpin RNA-targeting PTEN. The impact of PTEN knockdown on sensitivity to radiation in the presence or absence of PTEN downstream signaling inhibitors was investigated. PTEN knockdown conferred acquired resistance not only to gefitinib but also to radiation on HCC827 cells. mTOR inhibitors alone failed to reduce HCC827 cell viability, regardless of PTEN expression, but ameliorated PTEN knockdown-induced radioresistance. PTEN knockdown-mediated radioresistance was accompanied by repression of radiation-induced cytotoxic autophagy, and treatment with mTOR inhibitors released the repression of cytotoxic autophagy to overcome PTEN knockdown-induced radioresistance in HCC827 cells. These results suggest that inhibiting mTOR signaling could be an effective strategy to radiosensitize NSCLC harboring the *EGFR* activating mutation that acquires resistance to both TKIs and radiotherapy due to *PTEN* loss or inactivation mutations. J. Cell. Biochem. 114: 1248–1256, 2013.

KEY WORDS: RADIOSENSITIVITY; NSCLC; PTEN

The development of epidermal growth factor receptor tyrosine kinase inhibitors (EGFR-TKI), such as gefitinib and erlotinib, has improved therapeutic efficacy for non-small-cell lung cancer (NSCLC). Although patients with NSCLC whose tumors harbor activating mutations in the *EGFR* show dramatic clinical responses to EGFR-TKIs [Lynch et al., 2004; Paez et al., 2004], all patients eventually develop resistance to EGFR TKIs. Resistance is often mediated by acquisition of the *EGFR* T790M mutation, which occurs in *cis* with the exons 18–21 activating mutation. Amplification of *MET* is the second most prevalent event for acquiring resistance to

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Young-Bin Lim and Cheol Hyeon Kim contributed equally to this work.

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EGFR-TKIs [Linardou et al., 2009; Wheeler et al., 2010]. In addition to these mutations, various other molecular mechanisms are involved in acquired resistance against EGFR-TKIs, including loss of phosphatase and tensin homolog (*PTEN*) [Kokubo et al., 2005; Wheeler et al., 2010]. Sos et al. recently reported that loss of *PTEN* contributes to gefitinib resistance by activating downstream EGFR signaling pathways, suggesting the therapeutic importance of PTEN expression in the treatment of NSCLC with EGFR-targeted drugs [Sos et al., 2009a]. As constitutive activation of the PKB/Akt and mTOR pathways induced by loss of *PTEN* confers gefitinib resistance in patients with NSCLC, the probable route to overcome loss of *PTEN*-mediated gefitinib resistance is pharmacological inhibition of the PKB/mTOR axis [Bjornsti and Houghton, 2004; Gridelli et al., 2008].

The mTOR signaling pathway integrates nutrient and mitogen signals to regulate cell proliferation, survival, autophagy, and angiogenic pathways and has been implicated in resistance to EGFR inhibitors [Bjornsti and Houghton, 2004; Gridelli et al., 2008]. Inhibitors of mTOR, such as rapamycin and everolimus, are being explored for treating NSCLC as single agents and in combination [Wang and Sun, 2009]. Although adding everolimus to gefitinib in patients with acquired resistance to EGFR TKI has been reported to overcome acquired resistance [Bianco et al., 2008], the frequent presence of intrinsic resistance against mTOR inhibitors in patients with NSCLS has limited clinical application of mTOR inhibitors [Wang and Sun, 2009; Carew et al., 2011].

Autophagy is a tightly regulated pathway involving lysosomal degradation of cytoplasmic organelles or cytosolic components. This pathway can be stimulated by multiple forms of cellular stress, including nutrient deprivation, hypoxia, reactive oxygen species, and DNA damage [Kroemer et al., 2010]. Autophagy mediates stress-induced metabolic adaptation and damage control allowing cells to survive in response to multiple stressors and helps defend against degenerative, inflammatory, infectious, and neoplastic diseases [Kroemer et al., 2010; Moreau et al., 2010]. In addition to its known cytoprotective function in response to various stressors, autophagy has the potential to contribute to cell killing in response to ionizing radiation (IR) as well as a variety of chemotherapeutic agents [Gewirtz et al., 2009; Zhuang et al., 2009]. Whether radiation-induced autophagy in cancer cells causes death or protects cells is controversial. In several studies, autophagy has been inhibited pharmacologically or genetically, resulting in contrasting outcomes of survival or death depending on the context. It seems that the outcomes are cell specific and highly dependent on the expression profiles of oncogenes and apoptosis regulating proteins [Zois and Koukourakis, 2009]. As most cancers have some deregulation of the apoptosis pathway, targeting autophagy pathways may produce better clinical outcomes in patients undergoing radiotherapy [Gewirtz et al., 2009; Zhuang et al., 2009].

In this study, we evaluated the efficacy of radiotherapy in NSCLC with acquired gefitinib resistance caused by PTEN deficiency to suggest radiotherapy as an alternative to EGFR TKIs. For this purpose, we generated HCC827 cells, an EGFR-TKI sensitive NSCLC cell line, expressing short-hairpin RNA (shRNA)-targeting PTEN using a lentiviral vector. Surprisingly, PTEN knockdown conferred acquired resistance not only to gefitinib but also to radiation on HCC827 cells. PTEN silencing-induced mTOR activation inhibited IR-induced autophagy, which determines radiation sensitivity of HCC827 cells. Thus, it is reasonable to speculate that an mTOR inhibitor would ameliorate PTEN deficiency-induced radioresistance in HCC827 cells by normalizing autophagy induction by IR. Here, we investigated the combined effect of mTOR inhibitors and radiotherapy to overcome the acquired resistance to radiotherapy caused by *PTEN* loss in NSCLC harboring *EGFR* activating mutation.

MATERIALS AND METHODS

CELL CULTURE

The NSCLC cell lines H1650 and HCC827 were obtained from the American Type Culture Collection (Manassas, VA). H1650, HCC827, PC-9, and HCC2279 cells harbor an *EGFR* exon 19 mutation (E746_A750del). H3255 cells harbor L858R mutation in *EGFR* exon 21. All cells were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (Lonza, Rockland, ME). All cells were grown at 37°C in a humidified atmosphere with 5% CO_2 and were in the logarithmic growth phase at the initiation of experiments.

DRUGS AND IRRADIATION

Gefitinib (cat #G-4408), RAD001 (cat # E-4040), and rapamycin (cat #R-5000) were purchased from LC Laboratories (Woburn, MA). Irradiation was performed using a ^{137}Cs γ-ray source (Atomic Energy of Canada) at a dose rate of 3.51 Gy/min at room temperature.

WESTERN BLOTTING

To prepare total cell lysates, cells were washed in ice-cold phosphate-buffered saline and lysed in 50 mm Tris, pH 7.4, containing 150 mm NaCl, 1 mm EDTA, 1 mm phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mm NaF, 1 mm sodium orthovanadate, 0.25% sodium deoxycholate, and 1% Nonidet P-40. The supernatants were collected after centrifugation at 10,000×*g* for 10 min. A 50-μg aliquot of protein sample was size-fractionated by electrophoresis and then transferred to a nitrocellulose membrane. The membranes were immunoblotted with primary antibodies and horseradish peroxidase-conjugated anti-mouse IgG. The immunoblotted proteins were visualized with an enhanced chemiluminescent system (Amersham Biosciences, Arlington Heights, IL). The primary antibodies were purchased from the following sources: anti-phospho EGFR (cat #2236), anti-PTEN (cat #9552), anti-phospho AKT (cat #4060), anti-phospho mTOR (cat #2971), anti-phospho S6 (cat #4856), anti-S6 (cat #2317), and anti-beclin1 (cat #3738) from Cell Signaling Technology (Danvers, MA); anti-AKT (cat #5298) and anti-EGFR (cat #03) from Santa Cruz Biotechnology (Santa Cruz, CA); and anti-LC3 (cat #NB100-2220) from Novus Biologicals (Littleton, CO).

LENTIVIRAL SHRNA PRODUCTION AND IN VITRO TRANSDUCTION

pLKO.1 lentiviral vectors expressing shRNA against PTEN (cat #25638) or scrambled shRNA (cat #1864) were purchased from Addgene (Cambridge, MA). The lentiviral vector stock was produced in 293T cells by transient cotransfection of an shRNA construct

together with pHR8.2R and pCMV-VSV-G helper constructs using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). The supernatants were harvested after 30 h and filtered through a 0.22-μm filter (Millipore, Vienna, Austria) to remove any nonadherent 293T cells. Next, HCC827 cells (5×10^4 in 35-mm-diameter culture dishes) were transduced with 1 ml of virus containing supernatant supplemented with 8 μg Polybrene. The medium was changed 1 day post-infection, and fresh medium was applied for 2 days.

MTT ASSAY

Cells in the logarithmic growth phase were harvested and plated in a 96-well plate, and gefitinib was added 24 h later. After a 3 day incubation, the MTT assay was performed to determine cell viability as follows: 4 h before the termination of each experiment, 50 μl of PBS containing 5 mg/ml MTT was added to the culture medium. The medium was then gently removed, and 50 μl of DMSO was added to each well to solubilize the formazan precipitate. The absorbance of each well was measured at 560 nm.

CLONOGENIC SURVIVAL ASSAY

Cells were trypsinized and plated on 60-mm dishes in triplicate 24 h before irradiation. The cells were treated with the indicated concentrations of mTOR inhibitors or vehicle control for 2 h prior to exposure to the indicated doses of radiation. The medium containing mTOR inhibitors was removed 6 h after radiation treatment, and cells were maintained in normal culture medium. Colonies were stained with crystal violet 14 days after the cells were plated, and colonies containing >50 cells were counted. Plating efficiency was calculated by dividing the average number of cell colonies per well by the number of cells plated. Surviving fractions were calculated by normalizing plating efficiency to appropriate control groups. PC-9, HCC2279, and H3255 cell lines were not clonogenic at plating densities of <2,000 cells/ml, and were thus not evaluated.

SMALL INTERFERENCE RNA AND CELL TRANSFECTION

Small interference RNA (siRNA) oligonucleotides were designed to interact with Beclin-1 or mTOR mRNA using the siRNA design tool provided by Dhamacon Research (Lafayette, CO). Oligonucleotide sequences were: 5′-GCTCAGTATCAGAGAGAA-3′ (Beclin1 siRNA), 5′-GTAAATGCTTCCACTAAAC-3′ (mTOR siRNA), and 5′-AAUCAA-CUGACUCGACCAC-3′ (scrambled siRNA). Transfections were carried out using the RNAiMAX protocol provided by Invitrogen. The transfected cells were used for subsequent experiments 24 h later.

RESULTS

PTEN REGULATED SENSITIVITY OF HCC827 CELLS TO RADIATION AND GEFITINIB

We selected a set of NSCLC cell lines for a detailed analysis of the relationship between PTEN activity and acquired resistance to gefitinib in lung cancer. HCC827 cells have a gefitinib-sensitizing EGFR-activating mutation (exon 19 in-frame deletion of amino acids E746–A750) and were derived from lung adenocarcinoma of a non-smoking woman (Fig. 1A) [Girard et al., 2000; Amann et al., 2005]. Therefore, HCC827 cells exhibit the clinicopathologic characteristics of NSCLC sensitive to EGFR TKIs. H1650 cells also have an exon 19 in-frame deletion of amino acids E746–A750 but additionally harbor a *PTEN* deletion mutation [Janmaat et al., 2006]. As previously reported [Helfrich et al., 2006], H1650 cells were gefitinib-resistant, but reconstitution of wild-type PTEN in H1650 cells by transient adenoviral expression sensitized the cells to gefitinib (Fig. 1A), emphasizing the therapeutic importance of PTEN expression to treat NSCLC with EGFR-targeted drugs. We next silenced PTEN in gefitinib-sensitive HCC827 cells with lentiviral shRNA (Fig. 1B). PTEN knockdown in HCC827 cells led to increased activity of PI3K downstream signaling molecules as evidenced by increased AKT and S6 phosphorylation when compared with that in the parental HCC827 and HCC827 cells expressing scrambled shRNA (Fig. 1B). In contrast to the gefitinib-sensitizing effect of reconstituting PTEN in PTEN-deficient H1650 cells, PTEN knock-down conferred gefitinib resistance to PTEN-proficient HCC827 cells, confirming *PTEN* loss as a gefitinib resistance mechanism in EGFR-mutant NSCLC. To determine whether PTEN deficiency affects sensitivity of the EGFR-mutant NSCLC to radiation treatment, clonogenic survival assays were performed with HCC827 cells stably expressing scrambled shRNA or shPTEN. As shown in Figure 1C, a significant increase in survival was observed in HCC827 cells expressing shPTEN as compared to those expressing scrambled shRNA. These data suggest that a PTEN deficiency can confer acquired resistance not only to gefitinib but also to radiation on EGFR-mutant NSCLC.

mTOR INHIBITORS AMELIORATED PTEN KNOCKDOWN-INDUCED RADIORESISTENCE IN HCC827 CELLS

The HCC827 cell is resistant to mTOR inhibitors, despite lacking known resistance mechanisms [Sos et al., 2009b; Moreira-Leite et al., 2010]. Consistent with these observations but despite complete inhibition of mTOR signaling by rapamycin treatment as evidenced by S6 dephosphorylation, HCC827 cells showed similar viability in response to a wide range of rapamycin concentrations, regardless of PTEN expression level (Fig. 2A). We next determined whether rapamycin could sensitize HCC827 cells to IR. As shown in Figure 2B, rapamycin sensitized HCC827 cells expressing scrambled shRNA. Interestingly, rapamycin also sensitized HCC827 expressing shPTEN, resulting in fewer surviving fractions, which was similar to the levels observed in HCC827 cells expressing scrambled shRNA. These observations suggest that mTOR might be the critical link mediating the PTEN deficiency in EGFR mutant NSCLC with acquired radioresistance (Fig. 1C). To confirm this hypothesis, we measured the effects of RAD001 treatment, another mTOR inhibitor, on PTEN knockdown-induced radioresistance using a clonogenic survival assay. Similar to rapamycin, RAD001 treatment led to no significant alterations in HCC827 cell viability, regardless of PTEN expression (Fig. 2C) but ameliorated PTEN knockdown-induced radioresistance (Fig. 2D).

BLOCKED AUTOPHAGY CONFERRED RADIORESISTANCE TO HCC827 CELLS

Amelioration of acquired radioresistance in HCC827 cells expressing shPTEN by mTOR inhibitors suggests that PTEN knockdown-

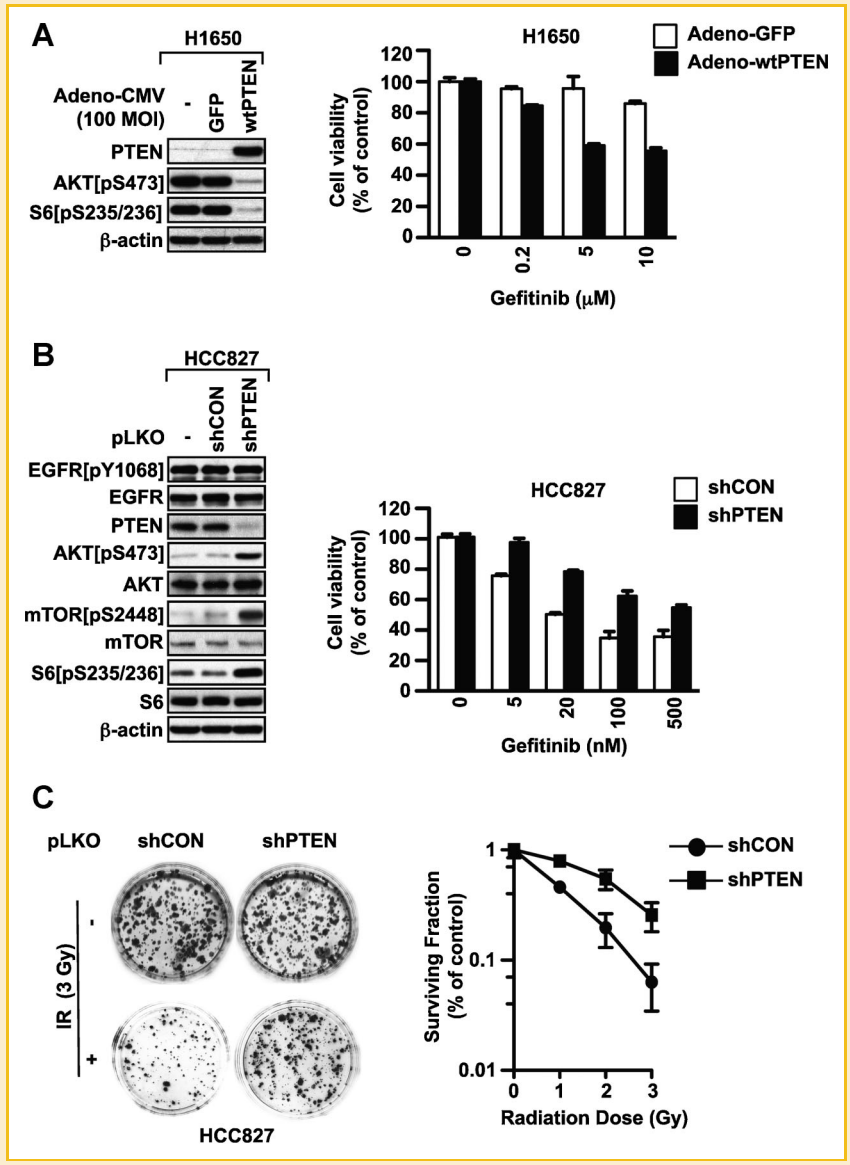

Fig. 1. Phosphatase and tensin homolog (PTEN) knockdown reduces sensitivity of HCC827 cells to radiation as well as gefitinib. A: H1650 cells were infected with adenoviral vector expressing GFP (GFP) or wild-type PTEN (wtPTEN) for 36 h, lysed, and PTEN and its downstream molecules were analyzed by Western blotting (left panel). H1650 cells were infected with adenoviral vector expressing GFP (Adeno-GFP) or wild type PTEN (Adeno-wtPTEN) for 36 h and then exposed to the indicated concentrations of gefitinib for 72 h. Cell viability was determined by the MTT assay (right panel). B: HCC827 parental cells (—) and HCC827 cells stably expressing scrambled short hairpin RNA (shCON) or shRNA targeting PTEN (shPTEN) were lysed and epidermal growth factor receptor (EGFR) and its downstream molecules were analyzed by Western blotting (left panel). HCC827 cells stably expressing scrambled shRNA (shCON) or shRNA targeting PTEN (shPTEN) were exposed to the indicated concentrations of gefitinib for 72 h. Cell viability was determined using the MTT assay (right panel). C: HCC827 cells stably expressing scrambled shRNA (shCON) or shRNA targeting PTEN (shPTEN) were exposed to the indicated doses of radiation and allowed to form colonies. Colonies containing >50 cells were then counted. The survival fraction was determined by dividing the plating efficiency of radiated cultures by the plating efficiency of nonradiated cultures. Black circles, shCON; black squares, shPTEN. Error bars, standard deviations (SDs) of three independent experiments in triplicate. Data shown represent a typical experiment or average values with SDs obtained from three independent experiments.

induced mTOR activation is mainly responsible for the acquired radioresistance of HCC827 cells expressing shPTEN. Radioresistance can be achieved by mTOR activation via multiple distinct mechanisms that have been described in NSCLC. One such mechanism is repressing autophagy by activating mTOR. To examine the possible involvement of deregulated autophagy in acquired radioresistance of HCC827 cells expressing shPTEN, we first checked whether autophagy regulation participates in determining radiosensitivity of HCC827 cells. During autophagy, the LC3-I protein is incorporated into the autophagosome membrane via coupling to phosphatidylethanolamine [Kroemer et al., 2010]. This lipidated form of LC3-I is called LC3-II. The electrophoretic mobility shift associated with conversion of LC3-I to LC3-II during autophagosome formation is detectable on LC3

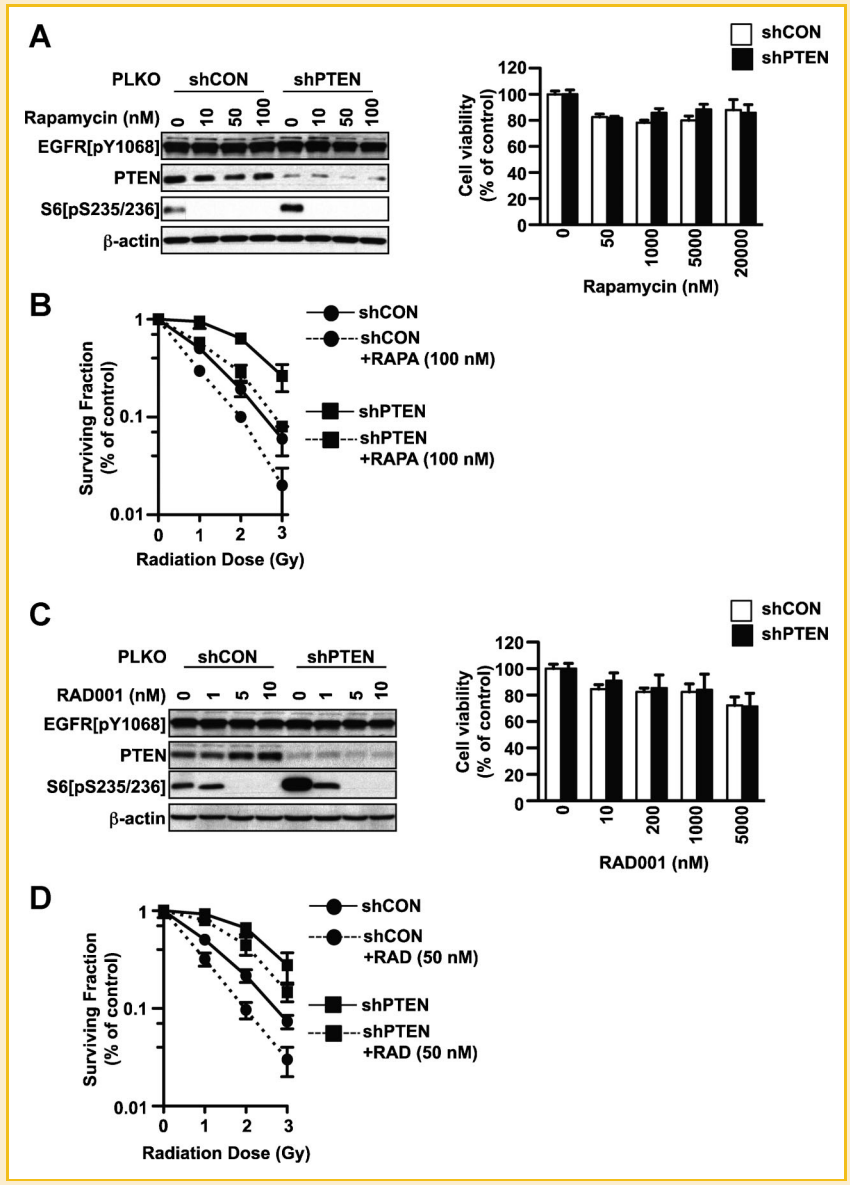

Fig. 2. mTOR inhibitors ameliorate phosphatase and tensin homolog (PTEN) knockdown-induced radioresistence in HCC827 cells. A: Immunoblots of HCC827 cell extracts stably expressing scrambled short hairpin RNA (shCON) or shRNA targeting PTEN (shPTEN) were treated for 2 h with the indicated concentrations of rapamycin and probed for the indicated proteins (left panel). HCC827 cells stably expressing scrambled shRNA (shCON) or shRNA targeting PTEN (shPTEN) were exposed to the indicated concentrations of rapamycin for 72 h. Cell viability was determined by the MTT assay (right panel). B: HCC827 cells stably expressing shCON (black circles) or shPTEN (black squares) were pretreated for 2 h with or without 100 nM of rapamycin (RAPA) and subsequently treated with the indicated doses of radiation, incubated in drug-containing media for another 6 h, and allowed to form colonies in drug-free medium. Colonies containing >50 cells were then counted. The surviving fraction was determined by dividing the plating efficiency of radiated cultures by the plating efficiency of nonradiated cultures. Continuous line, DMSO; broken line, 100 nM rapamycin. Error bars, standard deviations of three independent experiments in triplicate. C: Immunoblots of HCC827 cell extracts stably expressing scrambled shRNA (shCON) or shRNA targeting PTEN (shPTEN) were treated for 2 h with the indicated concentrations of RAD001 and probed for the indicated proteins (left panel). HCC827 cells stably expressing scrambled shRNA (shCON) or shRNA targeting PTEN (shPTEN) were exposed to the indicated concentrations of RAD001 for 72 h. Cell viability was determined by the MTT assay (right panel). D: HCC827 cells stably expressing shCON (black circles) or shPTEN (black squares) were pretreated for 2 h with or without 50 nM of RAD001 (RAD) and subsequently treated with the indicated doses of radiation then incubated in drug-containing media for another 6 h, and allowed to form colonies in drug-free medium. Colonies containing >50 cells were then counted. The surviving fraction was determined by dividing the plating efficiency of radiated cultures by the plating efficiency of nonradiated cultures. Continuous line, DMSO; broken line, 50 nM RAD001. Error bars, standard deviations (SDs) of three independent experiments in triplicate. The data shown represent a typical experiment or are average values with SDs obtained from three independent experiments.

Western blots and is an established autophagy biomarker [Mizushima, 2004; Mizushima and Yoshimori, 2007]. As shown in Figure 3A, IR significantly upregulated the expression of the autophagy marker LC3-II in HCC827 cells, demonstrating induction of autophagy by IR, and the IR-induced autophagy in HCC827 cells was completely blocked by short interfering RNA (siRNA)-mediated knockdown of beclin1, a crucial component of the autophagy cascade [He and Levine, 2010; Mizushima et al., 2010]. To assess

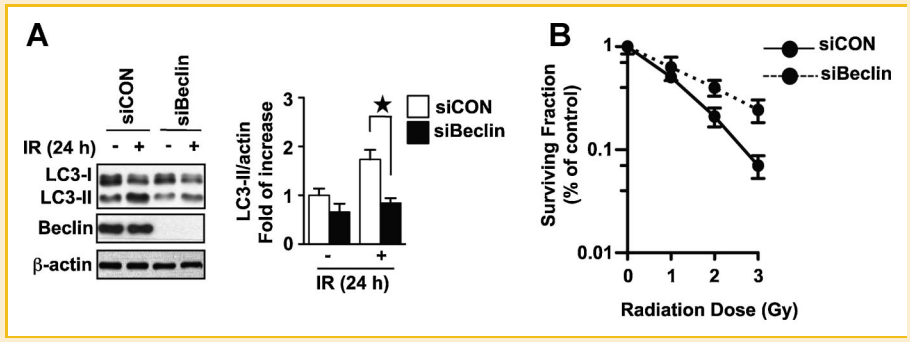

Fig. 3. Autophagy contributes to radiation sensitization of HCC827 cells. A: Approximately 24 h after transfection of scrambled siRNA (siCON) or beclin1 siRNA (siBeclin), HCC827 cells were treated with 6 Gy of ionizing radiation (IR). LC3-I and LC3-II expression was determined by Western blotting 24 h after radiation treatment, and β-actin was used as the loading control (left panel). Densitometric analysis and quantification of the expression of LC3-II versus β-actin in IR-exposed HCC827 cells (right panel). $^{*}P < 0.05$ compared to siCON and IR-treated cells (Student's *t*-test). B: HCC827 cells were treated with the indicated doses of radiation approximately 24 h after transfection of siCON (continuous line, black circles) or sibeclin (broken line, black circles). The colonies were stained with crystal violet 14 days after plating, and colonies containing >50 cells were counted. The surviving fraction was determined by dividing the plating efficiency of radiated cultures by the plating efficiency of nonradiated cultures. Error bars, standard deviations (SDs) of three independent experiments in triplicate. The data shown represent a typical experiment or are average values with SDs obtained from three independent experiments.

whether the induction of autophagy observed in IR-treated HCC827 was involved in determining radiosensitivity of HCC827 cells, we examined the effects of beclin1 knockdown on toxicity of IR to HCC827 cells using a clonogenic survival assay. As shown in Figure 3B, beclin1 knockdown by siRNA significantly decreased toxicity of IR to HCC827 cells, suggesting a negative contribution of IR-induced autophagy to survival of IR-exposed HCC827 cells.

mTOR INHIBITORS RELEASED REPRESSION OF CYTOTOXIC AUTOPHAGY TO OVERCOME PTEN KNOCKDOWN-INDUCED RADIORESISTANCE IN HCC827 CELLS

After demonstrating the negative contribution of IR-induced autophagy to survival of HCC827 cells, we next sought to determine possible involvement of deregulated autophagy in acquired radioresistance in HCC827 cells expressing shPTEN. Consistent with the results observed in parental cells (Fig. 3A), IR treatment resulted in increased LC3-II expression in HCC827 cells expressing scrambled shRNA, but, surprisingly, no significant change in HCC827 cells expressing shPTEN (Fig. 4A) was observed, raising the possibility that repression of cytotoxic autophagy is responsible for acquired radioresistance in HCC827 cells expressing shPTEN. Because mTOR activation was mainly responsible for acquired radioresistence in HCC827 cells expressing shPTEN (Fig. 2B and D) and the radioresistance shown in HCC827 cells expressing shPTEN was accompanied by deregulation of autophagy (Fig. 4A), we next examined whether mTOR knockdown with siRNA or treatment with mTOR inhibitors, which overcame PTEN knockdown induced-radioresistance (Fig. 2B and D), could release the repression of IR mediated-induction of cytotoxic autophagy in HCC827 cells expressing shPTEN. As shown in Figure 4B and C, mTOR siRNA or treatment with mTOR inhibitors resulted in significantly increased LC3-II expression in IR-exposed HCC827 cells expressing shPTEN. Collectively, these results suggest that PTEN knockdown-mediated mTOR activation inhibits cytotoxic autophagy induction by IR treatment to confer radioresistance to HCC827 cells. To confirm this hypothesis, we suppressed beclin1 expression using siRNA and measured its effects on mTOR inhibitor-induced radiosensitization observed in HCC827 cells using the clonogenic survival assay. As shown in Figure 4D, rapamycin radiosensitized HCC827 cells expressing shPTEN, and the radiosensitization was completely abolished by siBeclin1, clearly demonstrating the potential of an mTOR inhibitor to overcome PTEN knockdown-induced radioresistance, which was dependent on its ability to derepress inhibition of autophagy induction caused by PTEN knockdown.

DISCUSSION

In the present study, we found that PTEN knockdown reduced sensitivity of HCC827 cells to radiation as well as gefitinib and that PTEN knockdown-induced mTOR activation mediated the acquired resistance to radiation. This is the first report to show that PTEN deficiency confers acquired resistance to radiation and gefitinib on NSCLC cells harboring an *EGFR*-activating mutation. mTOR inhibitors clearly radiosensitized HCC827 cells, which have known resistance to mTOR inhibitors alone [Sos et al., 2009b; Moreira-Leite et al., 2010], suggesting that sensitivity to mTOR inhibitors does not determine the effectiveness of mTOR inhibitors with radiotherapy compared to radiotherapy alone for treating NSCLC harboring the *EGFR* activating mutation. mTOR is activated by the PI3K/Akt and mitogen activated protein kinase signaling pathways, which is often hyperactivated in many types of cancer. Thus, the mTOR axis has emerged as an attractive cancer therapeutic target [Bjornsti and Houghton, 2004; Gridelli et al., 2008]. Although the use of single-agent mTOR inhibitors has not been encouraging in clinical trials [Wang and Sun, 2009], they have been reported to function as radiation sensitizers in a breast cancer cell model and in other tumor xenografts in mice [Gewirtz et al., 2009]. Radiosensitization by mTOR inhibitors can occur via suppression of homologous recombination and nonhomologous end joining, two major

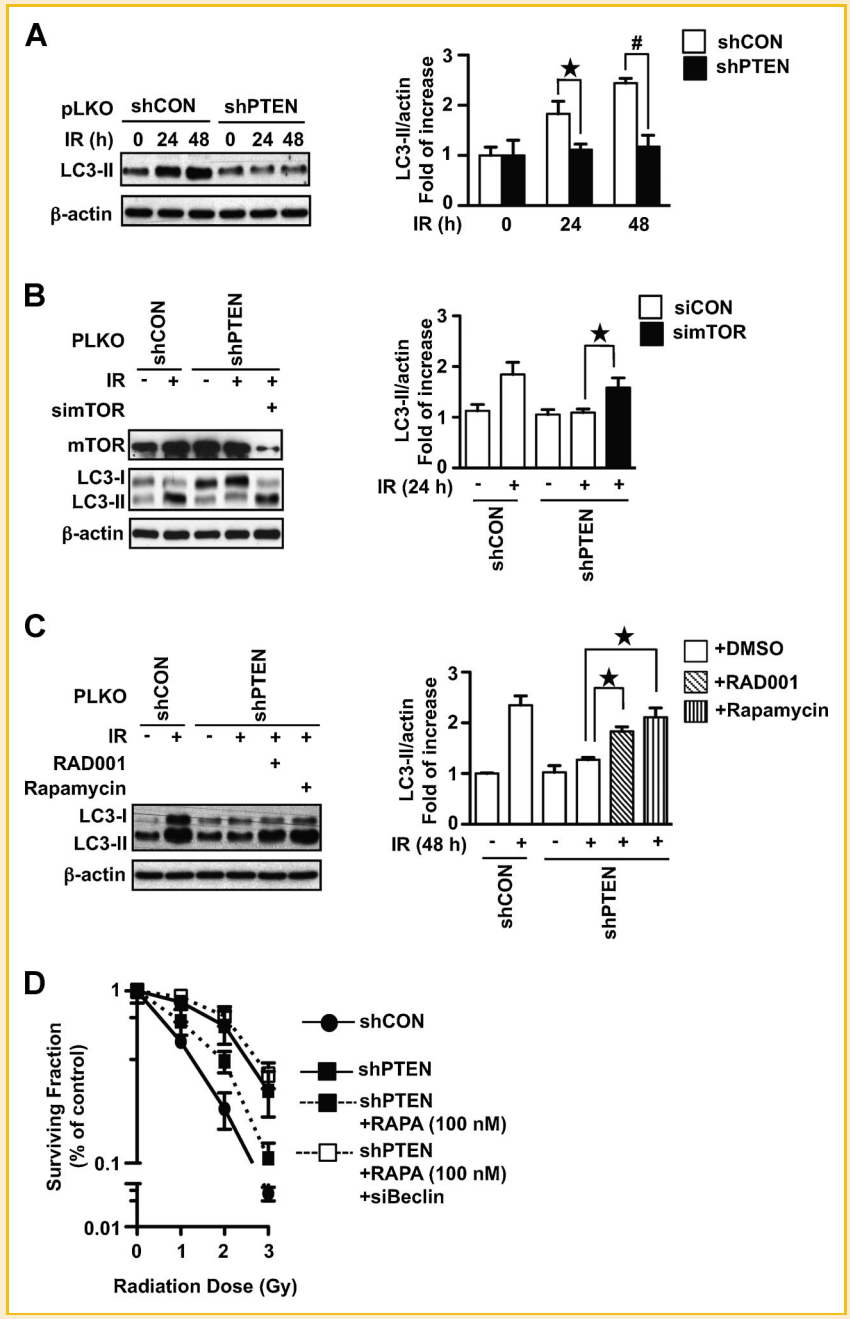

Fig. 4. mTOR inhibitors ameliorate phosphatase and tensin homolog (PTEN) knockdown-induced radioresistence by normalizing the induction of autophagy. A: Immunoblots of HCC827 cell extracts stably expressing scrambled short hairpin RNA (shCON) or shRNA targeting (shPTEN) were treated with 6 Gy of ionizing radiation (IR) for the indicated times and probed for LC3-II. β-actin was used as the loading control (left panel). Densitometric analysis and quantification of the expression of LC3-II versus beta-actin in IR-exposed HCC827 cells stably expressing scrambled shRNA (shCON) or shRNA targeting PTEN (shPTEN) (right panel). $^{*}P < 0.05$ compared to non-IR-treated controls (Student's *t*-test). B: Approximately 24 h after transfection of scrambled siRNA (siCON) or mTOR siRNA (simTOR), HCC827 cells stably expressing scrambled shRNA (shCON) or shRNA targeting PTEN (shPTEN) were treated with 6 Gy IR for 24 h. LC3-I and LC3-II expression was determined by Western blotting, and β-actin was used as the loading control (left panel). Densitometric analysis and quantification of the expression of LC3-II versus β-actin in IR-exposed HCC827 cells stably expressing scrambled shRNA (shCON) or shRNA targeting PTEN (shPTEN) (right panel). $^{*}P < 0.05$ compared to IR-treated shPTEN expressing cells (Student's *t*-test). C: HCC827 cells stably expressing scrambled shRNA (shCON) or shRNA targeting PTEN (shPTEN) pretreated for 2 h without or with the indicated concentrations of rapamycin or RAD001 were treated with 6 Gy IR for 48 h. LC3-I and LC3-II expression was determined by Western blotting, and β-actin was used as the loading control (left panel). Densitometric analysis and quantification of the expression of LC3-II versus β-actin in IR-exposed HCC827 cells stably expressing scrambled shRNA (shCON) or shRNA targeting PTEN (shPTEN) (right panel). $^{*}P < 0.05$ compared to non-IR-treated controls (Student's *t*-test). D: Approximately 24 h after transfection of scrambled siRNA or beclin1 siRNA (siBeclin), HCC827 cells stably expressing shCON (black circles) or shPTEN (black squares) were pretreated for 2 h without or with 100 nM rapamycin (RAPA) and then treated with the indicated doses of radiation, incubated in the drug-containing media for another 6 h, and allowed to form colonies in drug-free medium. Colonies containing >50 cells were then counted. The surviving fraction was determined by dividing the plating efficiency of radiated cultures by the plating efficiency of nonradiated cultures. Continuous line, DMSO; broken line, 100 mM rapamycin; black squares, shPTEN and siCON; white squares, shPTEN and siBeclin. Error bars, standard deviations (SDs) of three independent experiments in triplicate. The data shown represent a typical experiment or are average values with SDs obtained from three independent experiments.

mechanisms required for repairing IR-induced DNA double-strand breaks [Chen et al., 2011]. mTOR inhibitors also sensitize tumor cells by preventing radiation-induced expression of proangiogenic growth factors, which protect tumor microvasculature against radiation damage [Manegold et al., 2008]. Recent studies have identified autophagy as a mechanism for radiation sensitization mediated by mTOR inhibition [Gewirtz et al., 2009]. In the present study, IR significantly upregulated autophagy in HCC827 cells, and the IR-induced autophagy negatively contributed to survival of IR-exposed HCC827 cells. But, PTEN knockdown-mediated mTOR activation resulted in repression of cytotoxic autophagy to confer acquired radioresistance to HCC827 cells, and the repression of cytotoxic autophagy was released by mTOR inhibitor treatment to overcome PTEN knockdown-induced radioresistance in HCC827 cells. NSCLCs harboring EGFR activating mutations, including HCC827 cells, exhibit delayed double-strand DNA break (DSB) repair kinetics compared with wild-type EGFR NSCLCs in response to IR, and the mutant EGFR-expressing NSCLC cell lines lack the ability to block DNA synthesis in response to IR [Das et al., 2006]. In mutant EGFR-expressing cell line, constitutive activity and signaling from the EGFR likely promotes progression through IR-induced cell cycle checkpoints and thereby reduces DNA repair. In yeast, activation of ATF1/ATG13 kinase complex initiates autophagy. This mechanism of autophagy initiation is conserved, as unc-51-like kinase 1 (ULK1) and unc-51-like- kinase 2 (ULK2) are two mammalian orthologs of yeast ATF1 and form similar complex with mammalian ATG13 [Mizushima, 2010]. p53 inhibits autophagy through a transcription-independent effect exerted from a cytoplasmic localization [Tasdemir et al., 2008]. The autophagy-suppressor function of cytoplasmic p53 can be reversed by K386 sumoylation and K120 acetylation [Naidu et al., 2012]. p53 may potently induce autophagy through transcriptional activation of the autophagy-inducing proteins. ULK1 and ULK2 are transcriptional targets of p53, and their upregulation by p53 leads to elevated autophagy in response to DNA damage and contributes to subsequent cell death [Gao et al., 2011]. High mTOR activity prevents ULK1 activation by phosphorylating ULK1 [Jung et al., 2009]. Thus, PTEN loss and following elevated mTOR activity may contribute to increased clonogenic survival in HCC827, which harbors wild-type p53, when exposed to IR by repressing ULK1 pathway, which regulates p53-induced cytotoxic autophagy. Therefore, our data suggest that inhibition of mTOR signaling could be an effective strategy to radiosensitize NSCLC harboring the *EGFR* activating mutation that acquires resistance to both TKIs and radiation because of *PTEN* loss or inactivating mutations.

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