CANCER BIOLOGY & THERAPY
2016, VOL. 17, NO. 10, 1070–1078
http://dx.doi.org/10.1080/15384047.2016.1219818

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RESEARCH PAPER

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Downregulation of a novel human gene, ROGDI, increases radiosensitivity in cervical cancer cells

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ABSTRACT

ROGDI is a protein that contains a leucine zipper domain and may be involved in cell proliferation. In addition, ROGDI is associated with genome stability by regulating the activity of a DNA damage marker, γ-H2AX. The role of ROGDI in tumor radiosensitization has not been investigated. Previous studies have indicated that radiosensitivity is associated with DNA repair and the cell cycle. In general, the G2/M DNA damage checkpoint is more sensitive to radiation, whereas the G1/S phase transition is more resistant to radiation. Inhibition of cyclin-dependent kinases (CDKs) can lead to a halt of cell cycle progression and a stay at different phases or checkpoints. Our data show that the downregulation of ROGDI led to a decreased expression of CDK 1, 2, cyclin A, B and resulted in a G2/M phase transition block. In addition, the downregulation of ROGDI increased cell accumulation at the G2 phase as detected using flow cytometry and decreased cell survival as revealed by clonogenic assay in HeLa and C33A cells following irradiation. These findings suggest that the downregulation of ROGDI can mediate radiosensitivity by blocking cells at G2/M, the most radiosensitive phase of the cell cycle, as well as exerting deleterious effects in the form of DNA damage, as shown by increased γ-H2AX activation.

Abbreviations: CDKs, Cyclin-dependent kinases; GBM, glioblastoma multiforme; ATM, ataxia-telangiectasia mutation; CCK-8, Cell Counting Kit-8; PI, propidium iodide; PLA, *In situ* proximity ligation assay; DSB, double strand break

ARTICLE HISTORY
Received 11 May 2016
Revised 15 July 2016
Accepted 29 July 2016

KEYWORDS
γ-H2AX; cyclin-dependent kinases; DNA; damage; ROGDI; radiosensitivity

Introduction

Radiation therapy is widely used in many cancer treatments, but some patients may suffer from local recurrence or distant metastasis after irradiation. Thus, identifying the mechanisms underlying tumor cell radioresistance may improve the outcome of cancer therapies. Clinical observations in radioresistance are as follows: cervical adenocarcinoma has a lower radiosensitivity than cervical squamous cell carcinoma,[1] tumor hypoxia and necrosis influence radioresistance,[2] and limited therapeutic effectiveness can be achieved by radiation-only therapy in some non-epithelial cancers such as glioblastoma multiforme (GBM), melanoma, and soft-tissue sarcoma.

Factors associated with the cell cycle and DNA damage repair are implicated in radiosensitivity.[3] Generally, cells at the G2/M phase transition have higher radiosensitivity, whereas cells at the G1/S are more radioresistant, possibly because cells in the G2/M phase transition cannot undergo DNA repair before entering mitosis, resulting in mitotic catastrophe.[3,4] Critical genes involved in DNA damage repair are ataxia–telangiectasia mutation (ATM), p53, and p21.[5,6,7] The activation and elevation of p53 can lead to 2 results: arrest of the cell cycle at G1 or G2 phase or apoptotic cell death. Cells can either repair DNA damage in the G1 phase or die from unrepairable excessive DNA damage.[8] Cells can repair damage in the G1 phase, or cells with excessive damage could be removed from the organism (G2). Radiation damage to DNA leads to elevation of p53 protein expression, which in turn induces the expression of the downstream regulatory factor, p21, and halts the cell cycle through the cyclin-dependent kinase inhibitor (CDKI) mechanism. The progression of cell cycle resumes after DNA repair. Tumor cells treated with radiation may also relapse through this mechanism.[5,6,7] In addition, the PI3K[9,10,11] and ERK[12,13] signaling pathways can enhance DNA repair after radiation therapy. Interventions via these pathways may increase radiosensitivity.

ROGDI, the *Homo sapiens* rogdi homolog (*Drosophila*), was identified during human renal endothelial cell screening in the NEDO human cDNA sequencing project. According to the NCBI Gene database (Gene ID: 79641), ROGDI is located on chromosome 16p13.3 with an 864-bp coding region, and encodes a protein of unknown function comprising 287 amino acids. Recently it has been found that a loss-of-function mutation of this gene causes Kohlschutter–Tonz syndrome.[14,15] Additionally, the NCBI GenBank predicts that the ROGDI

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gene product is a leucine zipper domain protein, suggesting that ROGDI is a transcription factor. The UniProt database predicts that ROGDI may act as a positive regulator of cell proliferation. In our laboratory, ROGDI expression was found significantly increased in many tumors and in the livers of mice with thioacetamide-induced early-stage liver fibrosis (data not shown), suggesting that ROGDI may play an important role in tumorigenesis and liver fibrosis. Recently, the GenomeRNAi Human Phenotypes database indicated that RNA interference (RNAi) of ROGDI increases γ-H2AX phosphorylation, suggesting that it may mediate genome stability.[16] Previous studies have revealed that γ-H2AX is a signaling molecule produced by DNA double-strand breaks and triggers a repair mechanism. In the present study, we investigated the association between ROGDI and radiosensitivity in cervical cells and a hypothesis that downregulation of the novel human gene ROGDI mediates the radiosensitivity of cancer cells after irradiation. The ROGDI gene was discovered serendipitously in the course of studying differentially expressed genes in the lipopolysaccharide-infused rat brain stem by suppression subtractive hybridization in our lab. ROGDI was one of the differentially expressed genes that we found. It is suggestive that this novel gene is stress-inducible and that radiation imposes strong stress on cells.

Results

Downregulation of ROGDI in various cell lines decreased cell proliferation

Given that ROGDI may be involved in cell proliferation, the present study used ROGDI small-hairpin-RNA-containing lentivirus to knock down ROGDI expression in various cancer cell lines (Fig. 1A). The CCK-8 and colony formation assay were used to test the cell proliferation rate after knockdown of ROGDI. Cancer cell proliferation rates in all 6 tested cancer cell lines decreased after ROGDI downregulation (Fig. 1B, 1C). To identify the role of ROGDI in the cell proliferation of cancer cells, we assessed ROGDI expression in different cells. We noted a strong effect in HeLa and C33A cells (Fig. 1B). Accordingly, these cell lines were chosen for the experiment.

Effects of ROGDI on cell cycle profile in cervical cancer cells

To identify the role that ROGDI plays in the cell cycle, we evaluated the growth-inhibitory effects of ROGDI knockdown in HeLa and C33A cancer cells by flow cytometry. HeLa and C33A cancer cells were arrested at the G2/M phase by ROGDI knockdown (Fig. 2A). It is noteworthy that more cells accumulated at G2 phase in the ROGDI knockdown group than in the shLacZ group at 24 h after cell synchronization(Fig. 2A). This observation suggests that downregulation of ROGDI may influence radiosensitivity and consequently accumulation of cells at the G2 phase. However, factors that affect radiosensitivity are associated with DNA damage repair and the cell cycle, and generally, cells arrested at the G2 phase show higher radiosensitivity owing to high genomic instability. CDK has little kinase activity; only the cyclin–CDK complex is an active kinase.[17] Our results showed that downregulation of ROGDI decreased the expression of CDK1, 2, cyclin A and B; however, Cyclin E was unaffected by the ROGDI knockdown (Fig. 2B). The CDK2–cyclin A complex causes cells to continue through the S phase or transition from G2 to the M phase,[18,19,20] and is required for entry into the M phase.[20] Cyclin A remains associated with CDK1 from late S into late G2 phase, when it is replaced by cyclin B; therefore, we expect the downregulation of ROGDI to increase cell radiosensitivity owing to G2/M phase arrest (Fig. 5). The concomitant decrease in CDK2/cyclin A and CDK1/cyclin B and increase in $p53^{WT}$ and p21 (Fig. 2B) led to cell cycle arrest at the G2/M cell cycle checkpoint and enhanced radiation-induced DNA damage in cervical cancer cells.

Downregulation of ROGDI increased radiosensitivity of HeLa and C33A cells

A clonogenic assay was performed to evaluate the effect of downregulation of ROGDI in HeLa and C33A cervical cancer cells. HeLa and C33A cells were infected with control scramble or siRNA (Fig. 3E) for 48 h before 0, 2, 4, 6 Gy of irradiation. Significantly lower surviving fractions were noted in HeLa and C33A cells with ROGDI knockdown (Fig. 3A, 3B) than in the control-scramble-infected cells ($^{**}P < 0.01$). These results showed that downregulation of ROGDI sensitizes cervical cancer cells to a radiation-induced decrease in cell survival after radiation. In view of these results, we speculated that the inhibition of CDK1, 2 in combination with mediated ROGDI in HeLa and C33A cells decreases the effect of ROGDI-mediated sensitivity to radiation-induced cell death. To assess whether the radioresistant effect of ROGDI was dependent on CDK1,2, this study to compare the survival curve in with/without ROGDI cancer cells with and without CDK1,2 inhibitor. Because inhibition of CDK1, 2 by AZD5438 resulted in an increase of ionizing radiation (IR)-induced apoptosis in 3 non-small cell lung cancer (NSCLC) cell lines by reduced DNA double-strand break (DSB) repair by HR,[21] we treated downregulation of ROGDI HeLa and C33A cells with AZD5438 (150 nM) for 24 h and treated with IR as indicated. A clonogenic assay was performed to evaluate scramble HeLa and C33A cells with AZD5438 (150 nM) showed enhanced radiation-induced cell death and the effect of ROGDI downregulation in HeLa and C3AA cells with or without AZD5438 did not alter clonogenic survival as we had expected (Fig. 3C, 3D). These results showed that ROGDI mediates resistance to radiation-induced cell death through CDK1, 2.

Phosphorylated γ-H2AX (Ser139) was expressed rapidly in ROGDI downregulation in HeLa and C33A cells after irradiation

Expression of the sensing/signaling molecule of DNA damage, phosphorylated γ-H2AX, was increased after irradiation of cervical cancer cells (Fig. 4A). γ-H2AX is a marker of DNA damage. These results show that downregulation of ROGDI leads to imbalance of major cell cycle regulators, such as CDKs and/or cyclins. Decreased CDK1, 2 and cyclin A, B caused by ROGDI knockdown arrests the cell

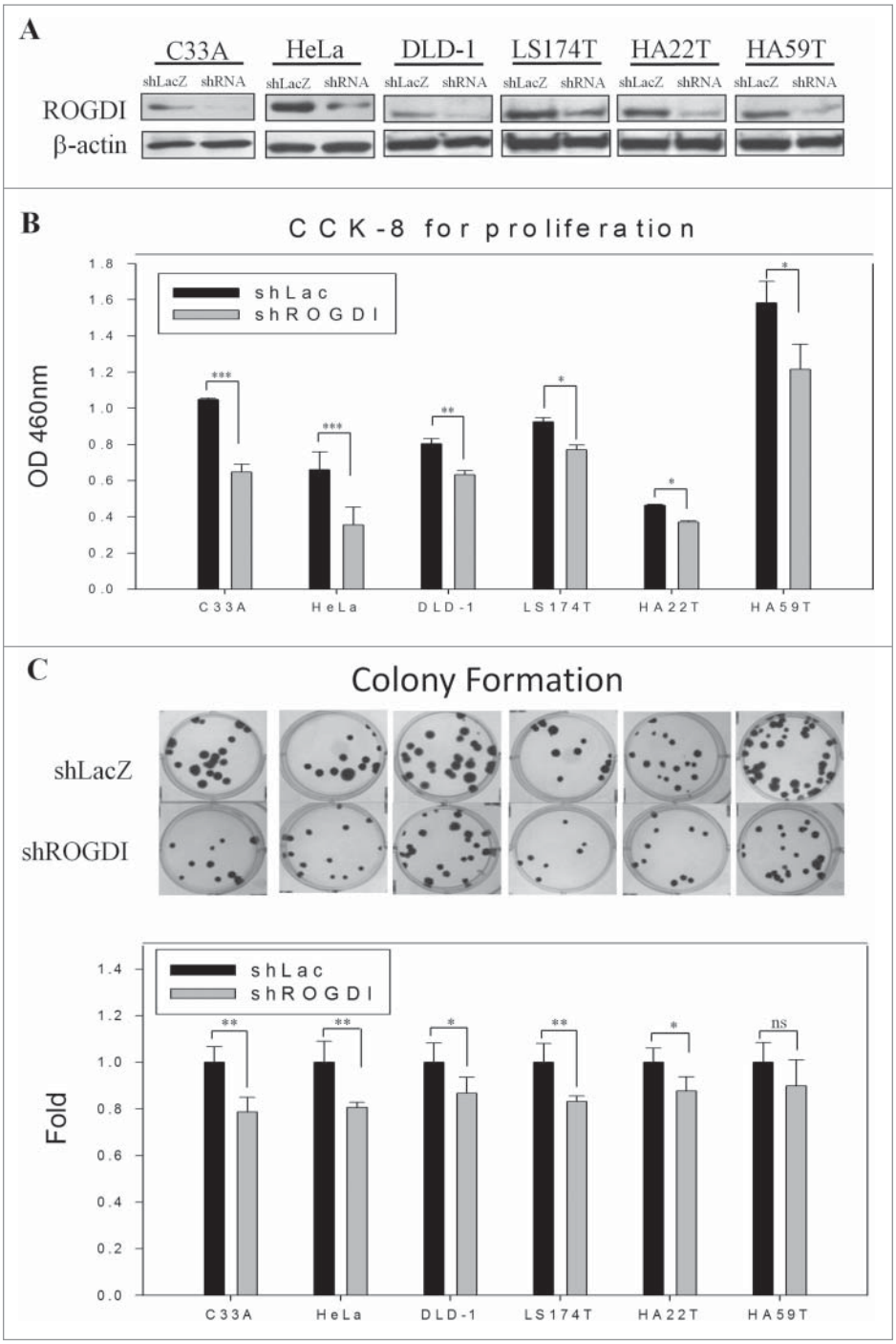

Figure 1. Effects of ROGDI knockdown on tumor cell proliferation. (A) Western blotting indicated knockdown of ROGDI protein levels by shRNA in various cell lines. shLacZ, off-target control. Effects of ROGDI knockdown on cell proliferation were assessed by CCK-8 proliferation assay (B) and colony formation assay (C) The error bar represents the standard error of the mean: $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

cycle at the G2/M checkpoint, which, in combination with the activation of γ-H2AX, results in genomic DNA destabilization. Induction of genomic DNA destabilization by downregulation of ROGDI coupled with upregulation of $p53^{WT}$ and p21 ultimately resulted in a decreased survival fraction of cervical cancer cells post-radiation. Our results showed the expression levels of γ-H2AX in HeLa and C33A cells after 0, 10, 20, 30, and 60 m of 6 Gy after irradiation (Fig. 4A). We observed that downregulation of ROGDI cells had more serious damage at 10 m than non-regulated cells (Fig. 4A, 4B). Thus, ROGDI may be a protein has a significant effect on DSB of DNA after irradiation. However, the genome seemed to be broken more easily after irradiation in ROGDI-downregulated HeLa and C33A cells. Due to the γ-H2AX expression, it is suggesting that radiosensitivity induced by the reduction of ROGDI expression was a result of decreased CDKs 1, 2; cyclins A, B and increased p21. In the aspect of long term γ-H2AX expression following irradiation, the expression of γ-H2AX did not differ between cancer cells with and without ROGDI modulation after 4 h of irradiation. However, after 24 h of irradiation, attenuated expressions of γ-H2AX were

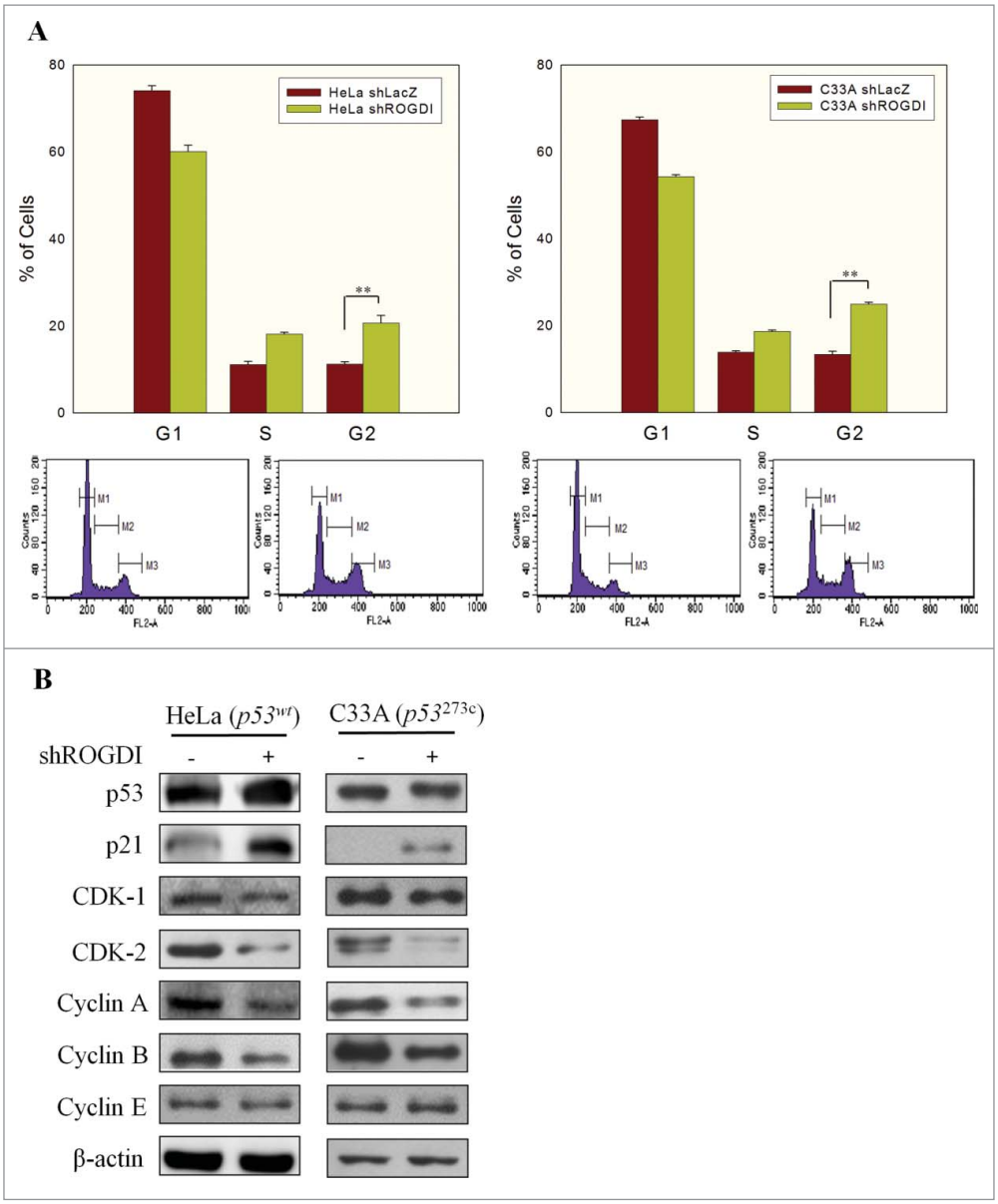

Figure 2. Cell cycle profile (A) and expression of cell cycle regulators (B) in HeLa and C33A cells treated with shROGDI for 24 h.

observed in the scrambled RNA-transfected cells compared with the ROGDI-silenced HeLa and C33A cells (Fig. 4C). Thus, cell cycle arrested at the G2/M phase, in combination with the activation of γ-H2AX, and induce great genomic DNA damage and causing immediately cell death.

Discussion

To examine the expression of ROGDI and major cell-cycle related proteins in different phases. Interestingly, we discovered that ROGDI and CDK2/cyclin A were concomitantly expressed. Knockdown of ROGDI leads to cell accumulation at the G2 phase as well as upregulation of the $p53^{WT}$–p21 axis of the G2/M checkpoint pathway; however, $p53^{273c}$ was unaffected by ROGDI knockdown. The result demonstrates that p21 is more important factor rather than p53. On the whole, the ROGDI knockdown-induced cell cycle arrest and upregulation the of $p53^{WT}$–p21 axis renders cells more susceptible to a radiation-induced decrease in survival by imposing additional stress on the cells.

We were interested in the mechanism underlying ROGDI mediation of radioresistance. p21 and p27 are among the molecules associated with cell cycle arrest,[22,23] with respect to the correlation between irradiation and oncogenes, $p53^{WT}$ may select G1 arrest pathways or apoptosis,[24] and our results showed that ROGDI downregulation led to increased $p53^{WT}$ and p21 protein levels. We accordingly speculate that ROGDI plays a role in radioresistance. We demonstrated that the expression of the major cell cycle players CDKs 1, 2; cyclins A, B was downregulated when ROGDI was downregulated. In contrast, activated γ-H2AX was time-dependently upregulated following 6 Gy irradiation in both cervical cancer cell lines. More importantly, we found that downregulation of ROGDI and activation of the DNA DSB marker γ-H2AX in its phosphorylated form of phospho-γ-H2AX was increased after irradiation. Previous studies have shown that almost every DNA

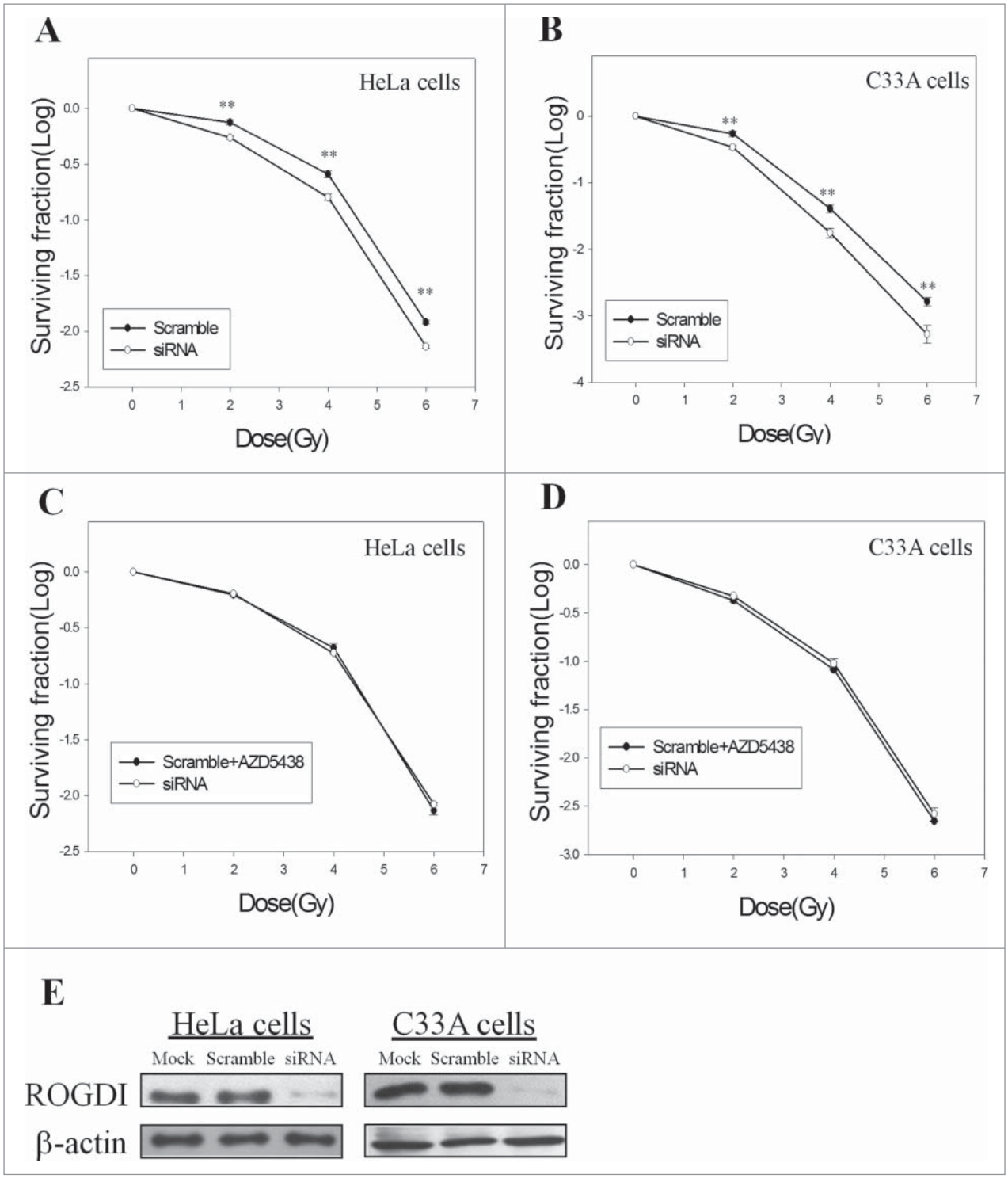

Figure 3. ROGDI knockdown enhanced cells to radiation-induced cell death. Clonogenic survival curves of HeLa cells (A) and C33A cells (B) treated with siROGDI and scramble siRNA. Knockdown of ROGDI enhances radiation-induced cell death. Clonogenic survival curves were compared between the siROGDI and scramble in C33A cells. (C) (D) HeLa and C33A cells treated with AZD5438(150 nM) showed similar clonogenic survival curves with cells treated with siROGDI. The error bar represents the standard error of the mean: $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

DSB forms a γ-H2AX focus. γ-H2AX is required for p21-induced cell cycle arrest[25] and *in vivo* $p53^{WT}$ is essential for determining radiosensitivity in a variety of tissues.[26,27,28] Our study using *In situ* proximity ligation assay (PLA) and γ-H2AX expression revealed that ROGDI downregulation attenuated the repair of DNA damage in both HeLa and C33A cells. In addition, 24-hour γ-H2AX expression of ROGDI knockdown or not (Fig. 4C) correlated corresponding clonogenic survival (Fig. 3A and 3B). The data is consistent with a study showing radiosensitivity with residual damage, measured at longer times after radiation.[29] As expected, our results showed that knockdown of ROGDI leads to the downregulation of CDKs 1, 2; cyclins A, B and consequently upregulation of $p53^{WT}$ and p21 proteins and activation of γ-H2AX after irradiation. These

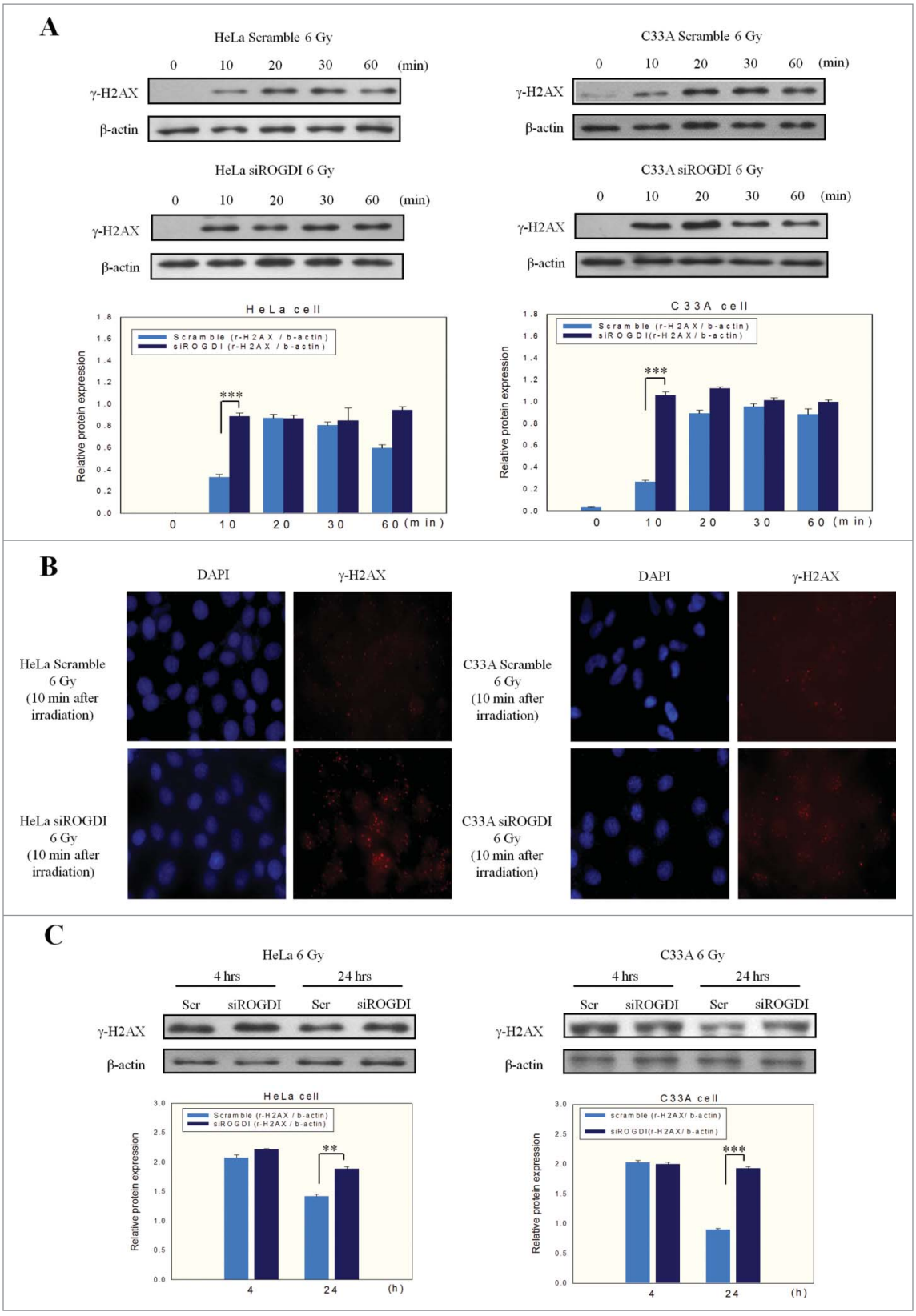

Figure 4. ROGDI knockdown enhanced γ-H2AX expression in HeLa and C33A cells treated with irradiation. (A) Expression of γ-H2AX was measured using western blot in HeLa and C33A cells after irradiation with 6 Gy for 0, 10, 20, 30, or 60 min. (B) Fluorescent immunodetection of γ-H2AX foci in HeLa and C33A cells irradiated with 6 Gy for 10 min. (C) Expression of γ-H2AX was measured using protein gel blot in HeLa and C33A cells after irradiation with 6 Gy for 4 or 24 h. The error bar represents the standard error of the mean: $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

results constitute a strong mechanistic explanation for the observation that ROGDI downregulation decreased survival fractions in the clonogenic assay.

The specificity of the sensitizing effect of radiation-induced cell death could be elucidated by a clonogenic assay. The induction or suppression of radiation-induced cell death by ROGDI

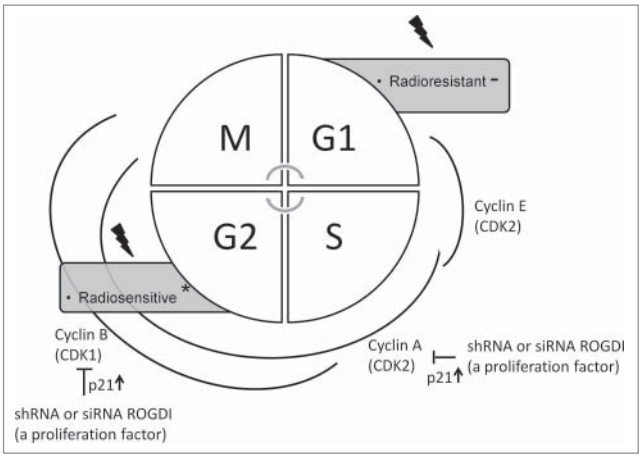

Figure 5. A proposed model of the roles of ROGDI in cell cycle progression and radiosensitivity of the cell. ROGDI promotes cell cycle progression by inhibiting p21 expression and enhancing CDK/cyclin complexes formation. Knockdown of ROGDI results in G2/M arrest and sensitizes cells to irradiation.

suggests an important role of ROGDI in tumorigenesis and the cell death mechanism in cervical cancer cells. Additional ROGDI-associated proteins unique to HeLa and C33A or other cells may be important for determining cell survival after irradiation. Future isolation and characterization of these proteins will provide insight into the potential treatment of human cervical cancers by radiation therapy.

Materials and methods

Cell culture and antibodies

The HeLa cell line (BCRC-60005), C33A cell line (BCRC-60554), DLD-1 cell line (BCRC-60132), LS174T (BCRC-60053), HA22T (BCRC-60168), and HA59T (BCRC-60169) were purchased from the Bioresource Collection and Research Center (BCRC), Hsinchu, Taiwan. HeLa, C33A, and LS174T Cells were maintained in minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS), 100-U/mL streptomycin, and 100-mg/ml penicillin. The DLD-1 cells were maintained in Roswell Park Memorial Institute medium supplemented with 10% FBS, 100-U/mL streptomycin, and 100-mg/ml penicillin. HA22T and HA59T were maintained in MEM supplemented with 10% FBS, 100-U/mL streptomycin, and 100-mg/ml penicillin (all from Gibco). Primary antibodies targeting the following proteins were used: CDK1, CDK2, cyclin A, cyclin B, cyclin E, p53, and corresponding secondary antibodies (goat anti-rabbit IgG and goat anti-mouse IgG) (all from Santa Cruz); β-actin, ROGDI, p21, γ-H2A.X (phospho S140) and γ-H2A.X (phospho S139) (all from Abcam); and ROGDI (Proteintech, 17047-1-AP).

Stable knockdown of ROGDI in cell lines

All recombinant lentiviruses (GFP C6-4-2, negative cloning vector C6-4-3, and ROGDI TRCN0000356592) were purchased from the RNAi Core Facility, Academia Sinica, Taiwan. Lentiviral infection (MOI 0.3) was performed according to manufacturer's protocol. Lentivirus-transduced cells were selected in media containing 0.2–2 μg/ml of puromycin.

Short interfering RNA (siRNA)

siRNAs (Santa Cruz, sc-93538) were used to silence *rogdi* according to the protocol provided by the manufacturer. siRNA (7.5 μl) and 250-μl Opti-MEM (Gibco, 11058021) were mixed. Lipofectamine 2000 (Invitrogen, 11668-019) (7.5 μl) was added to the other Opti-MEM (250 μl) mixture and mixed for 5 min. The diluted siRNA and Lipofectamine were mixed for 20 min. The reagents were added into 6-well plates in which cells had been seeded (5×10^5 cells/well) for 16 h. Control cells were treated with Stealth RNAi Negative Control Duplex (Invitrogen, 12935300).

Western blot analysis

Cellular lysates were obtained by lysing cells in radioimmunoprecipitation assay buffer containing protease and phosphatase inhibitors. Thirty micrograms of lysates were resolved on 10% or 12% sodium dodecyl sulfate-polyacrylamide gels and transferred onto polyvinylidene fluoride membranes. Western blotting to assay CDK1, CDK2, cyclin A, cyclin B, cyclin E, p21, p53, γ-H2A.X (phospho S139), and β-actin was performed.

Cell proliferation and colony formation assay

Cell proliferation was analyzed using a Cell Counting Kit-8 (Sigma-Aldrich, 96992–500) according to the manufacturer's instructions. The amount of water-soluble formazan dye was determined by measuring the absorbance of reduced salt WST-8 at 460 nm, which is proportional to the number of viable cells in the medium. For the colony formation assay, cells (20–50/well, according to the cell characteristics) were plated in 6-well plates. In the 1 to 2 weeks following seeding, glutaraldehyde (6.0% v/v) and crystal violet (0.5% w/v) were added to fix and stain the colonies, respectively.

Cell cycle phase detection by flow cytometry analysis

Cells were trypsinized, washed with cold phosphate-buffered saline, and resuspended in cold ethanol. After centrifugation, the supernatant was removed and a solution containing 50 μg/ml of propidium iodide (PI) and 100 μg/ml of ribonuclease A was added. The cell suspension was analyzed on a flow cytometer.

Irradiation and clonogenic assay

Cells treated under different conditions (siRNA or scramble) were irradiated in 25 T flasks. According to the dose of 0, 2, 4 and 6 Gy, cells (100, 400, 1600, and 6400) were plated in 6-well plates immediately following irradiation, respectively. One to 2 weeks after irradiation, we used glutaraldehyde (6.0% v/v) and crystal violet (0.5% w/v) to fix and stain colonies, respectively. Cells were counted by a stereomicroscope. A colony was defined to be surviving when at least 50 cells were counted.

In situ proximity ligation assay (PLA)

To investigate the phosphorylated form of phospho-γ-H2AX, we used the Duolink reagent kit (SIGMA -ALDRICH, DUO92102). We seeded 1×10^3 cells in 200 ml of medium into a chamber slide for culture. Cells were fixed in 1%

paraformaldehyde for 15 min after 6 Gy of irradiation for 10 min and then washed twice with Wash Buffer A for 5 min. Cells were incubated with a blocking solution at 37°C for 30 min, and then washed twice with Wash Buffer A for 5 min. The procedures for administrating the primary antibodies (Incubate with phospho-γ-H2AX antibodies from 2 different species), PLA probes, hybridization, ligation, amplification, detection, and mounting were performed according to the manufacturer's recommended protocol. The cells were observed using a fluorescence microscope (Axio Observer Z1, Carl Zeiss MicroImaging, Inc., Welwyn Garden City, UK), and photographed using an integrated camera with the appropriate filter for detection.

Statistical analysis

A comparison of the clonogenic assay of each pair was performed using a paired *t*-test. A *P* value of <0 .05 was considered statistically significant. Statistical analyses were performed using Sigma plot v10 (Systat Software, CA, USA).

Disclosure of potential conflicts of interest

The authors declare no conflicts of interest.

Funding

This study was partially supported by "Aim for the Top University Plan" of the National Sun Yat-sen University, NSYSU-KMU Joint Research Project (#NSYSUKMU 105-P012) and by a grant for the Chang-Gung Medical Research Project (CMRPG8C0501).

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