

Lentiviral vector with a radiation-inducible promoter, carrying the ING4 gene, mediates radiosensitization controlled by radiotherapy in cervical cancer cells

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Abstract. The presence of hypoxia in solid tumors is considered one of the major factors that contribute to radiation resistance. The aim of the present study was to establish a therapeutic system, which can be controlled by radiation itself, to enhance radiosensitivity. For this purpose, a lentiviral gene therapy vector containing the human inhibitor of growth 4 (ING4) and its upstream promoter, human early growth response factor-1 (EGR1), which possesses the radiation-inducible characteristics to activate the transcription of its downstream genes, was constructed. Downstream fluorescence proteins were investigated to ensure that the EGR1 promoter was induced by irradiation. Furthermore, ING4 open reading frame (ORF) expression was detected by western blotting. The cell cycle was analyzed by fluorescence-activated cell sorting analysis 48 h after the cells were exposed to X-rays ranging between 0 and 8 Gy. In cells stably and transiently transfected with reporter plasmids, the EGR1-driver gene was sensitive to ionizing irradiation. Furthermore, irradiation-induced ING4 gene expression was observed. The enhanced ING4 expression increased the number of cells in the G₂/M phase and decreased the proportion of cells in the G₁/S phase. Therefore, ING4 expression inhibited cell proliferation and was associated with less colonies being formed. Furthermore, ING4 suppressed hypoxia-inducible factor 1 α expression under hypoxic conditions and promoted cell apoptosis. Overall, these results revealed that combining the EGR1 promoter and ING4 ORF using a lentivirus system may be a promising therapeutic strategy with which to enhance radiosensitivity controlled by

radiation. However, further studies using *in vivo* models are required to confirm these findings.

Introduction

Cervical cancer is the fourth most common type of cancer among women worldwide, and the second most common in low- and middle-income countries according to the data from GLOBOCAN 2018, with >85% of new cases occurring in developing countries (1,2). Radiotherapy is widely used, particularly for locally advanced cervical cancer (3). Following cell cycle arrest, ionizing radiation-induced apoptosis will occur if DNA damage is not repaired (4). Strategies to enhance the expression levels of pro-apoptotic genes could be applied in gene-radiotherapy (5). Recently, gene-radiotherapy, which combines gene therapy with radiotherapy, has shown promising effects (6).

It has been demonstrated that the tumor suppressor inhibitor of growth 4 (ING4) is deleted in numerous types of cancer (7). ING4 serves an important role in cell proliferation, apoptosis, cell cycle arrest, migration and vascularization, and these are pivotal to tumor progression (7-11). The radiation-inducible early growth response 1 (EGR1) promoter, which includes six serum response elements sensitive to ionizing radiation (12,13), has attracted particular attention. Previous studies have indicated that the EGR1 promoter can enhance the expression of its downstream genes, such as TNF- α and IFN- γ (14,15).

During the process of tumor development, cells in hypoxia account for 10-50% of the tumor environment (16). Hypoxic tumor cells cause resistance to radiotherapy and chemotherapy, leading to tumor recurrence and distant metastasis (17). Hypoxia serves a vital role in angiogenesis (18). Furthermore, the hypoxia response element, upstream of the EGR1 promoter, can enhance the radiation-induced upregulation of therapeutic genes (19).

The present study combined the EGR1 promoter and ING4 open reading frame (ORF) as a cassette, which was integrated into HeLa cells to develop a cell line. Subsequently, the effects of ING4 on cell cycle arrest and cell proliferation were investigated when ING4 was induced using various doses of irradiation. Furthermore, the function of ING4 in hypoxia

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in HeLa cells was examined. Radiation and gene-combined treatment, termed gene-radiotherapy, exhibited a more prominent effect in HeLa cervical cancer cells *in vitro*.

Materials and methods

Cell culture and transfection. The human cervical carcinoma HeLa cell line was purchased from The Cell Bank of Type Culture Collection of Chinese Academy of Sciences and maintained at 37°C in DMEM (HyClone; Cytiva) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) and 100 U penicillin-streptomycin. The cells were grown in a humidified atmosphere of 5% CO₂ and 95% air.

HeLa cells in the exponential phase of growth were treated with trypsin at 37°C for 1 minute. After terminating digestion with 10% FBS, the cells were resuspended in complete culture medium. A total of 2x10⁵ cells were inoculated into each well of a 6-well plate and allowed to grow for 24 h prior to transfection. Subsequently, the cells in each well were transiently transfected with 2 µg p-enhanced green fluorescent protein (EGFP)-N1 (Sigma-Aldrich; Merck KGaA) or pEGR1-EGFP-N1 at room temperature (pEGR1-N1 was kindly provided by Dr Gerald Thiel from University of Saarland Medical Center, D-66421 Homburg, Germany) using 8.0 µl Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). The transfection mixture was replaced with fresh culture medium at 6 h after transfection and was irradiated at 24 h. The fluorescence images were captured at 72 h post-transfection using a fluorescence microscope (Nikon 80i; Nikon Corporation).

Construction of vectors. The mammalian cellular expression vector, pEGFP-N1 (6085-1), was purchased from Addgene, Inc. The CMV IE promoter was removed by endonucleases *AseI* and *EcoRI*. The promoter of human EGR1 ranging between -792 and 268 was isolated using primer pairs pEGR1-*AseI*-F/pEGR1-*EcoRI*-R and then directly cloned into the pEGFP-N1 vector, which was designated as pEGR1-EGFP-N1.

Similarly, the CMV promoter from construct pLV-mCherry(2A)puro (VL3405; Inovogen Tech. Co.) was replaced by the EGR1 promoter using primer pairs pEGR1-*Clal*-F/pEGR1-*EcoRI*-R and then used to develop the pEGR1-LV-mCherry(2A)puro vector. Furthermore, the human ING4 was cloned into pEGR1-LV-mCherry(2A)puro by *EcoRI* and *XhoI* with the hING4-*EcoRI*-F/hING4-*XhoI*-R primer pairs and the new construct was named pEGR1-LV-mCherry(2A)puro-hING4. Additionally, LV-mCherry(2A)puro-hING4 without any promoter was prepared.

Virus and cell lines. The recombinant lentivirus was obtained from HEK293T cells (Cell Bank of the Chinese Academy of Science) by transient transfection of lentivirus construct as well as helper plasmids psPAX2 (Addgene; cat. no. 12259) and MD2.G (Addgene; cat. no. 12260). Briefly, 1.0x10⁶ 293T cells were plated in a 6-cm dish and cultured for 18-24 h. The cells were then co-transfected with 1.7 µg pLV-mCherry(2A)puro, pEGR1-LV-mCherry(2A)puro, pEGR1-LV-mCherry(2A)puro-hING4 or LV-mCherry(2A)puro-hING4, 1.13 µg psPAX2 and 0.57 µg pMD2.G using

Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). 293T cells were maintained in DMEM medium with high glucose (Hyclone, cat. no. SH30243.01) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc; cat. no. 10099141) at 37°C in 5% CO₂. The supernatant containing the lentivirus was harvested at 48 and 72 h and then filtered through a 0.45-µm low protein-binding polysulfonic filter (EMD Millipore). HeLa cells were inoculated in a 6-well plate in advance at a density of 1.5x10⁵ cells per well and presented with ~60% confluency following incubation for 20 h. The cells were then infected with 1 ml lentivirus suspension in the presence of 8 µg/ml polybrene (Chemicon International; Thermo Fisher Scientific, Inc.). Following transduction for 48 h, HeLa cells were selected with 2.0 µg/ml puromycin for 10 days until all blank control cells were dead. The puromycin-resistant cells were stably infected with exogenous genes.

Cell cycle analysis. HeLa cells stably integrated with LV-mCherry(2A)puro-hING4 or pEGR1-LV-mCherry(2A)puro-hING4 were inoculated in a 6-well plate and were allowed to grow for 24 h. The cells were then exposed to X-rays with a dose ranging between 0 and 8 Gy (2 Gy gradient). Following growth for a further 48 h, the cells were treated with trypsin at 37°C for 1 min and subjected to centrifugation at 800 x g for 5 min at 4°C. After rinsing with 1X PBS, the cells were fixed in cold (4°C) 70% ethanol and stored at -20°C overnight. Following RNA removal using 50 µg/ml RNaseA (cat. no. R4875; Sigma-Aldrich; Merck KGaA) for 30 min at 37°C, cells were stained with 500 µg/ml propidium iodide (eBioscience™, Thermo Fisher Scientific, Inc.) at room temperature for 15 min. Subsequently, the cells were subjected to fluorescence-activated cell sorting (FACS)-based cell cycle analysis using a flow cytometer (FACSCalibur; BD Immunocytometry Systems) with CellQuest software (version 5.1). The percentages of cells in the sub-G₁ phase and different cell cycle phases were used as an index to evaluate the levels of cell apoptosis and cell cycle distribution, respectively.

Western blot analysis. Cells were lysed in 2X SDS buffer consisting of 0.1 M Tris-Cl, 0.2 M DTT, 4% SDS and 20% Glycerol and subsequently prepared for three cycles of boiling (95-100°C, 5-10 min) and cooling (4°C, 5-10 min). The proteins were quantified using the Bradford method and subsequently mixed with 0.2% Bromophenol blue. A total of 15 µg protein was loaded into each lane. Whole cell lysates were subjected to SDS-PAGE for protein separation and then electrophoretically transferred to a nitrocellulose membrane (Axygen; Corning Inc.), followed by blocking using PBS containing 5% fat-free milk. The nitrocellulose membranes were incubated with a rabbit polyclonal antibody against ING4 (cat. no. ab113425; Abcam), an antibody against hypoxia-inducible factor 1α (HIF-1α; cat. no. 14179; Cell Signaling Technology, Inc.) and a rabbit polyclonal antibody against β-actin (cat. no. 103030001; HarO, http://www.lifeqho.com/pd.jsp?id=6#_jcp=2) overnight at 4°C. Subsequently, the membranes were incubated with a HRP-conjugated rabbit IgG secondary antibody (cat. no. 7074; Cell Signaling Technology, Inc. <https://www.cellsignal>).

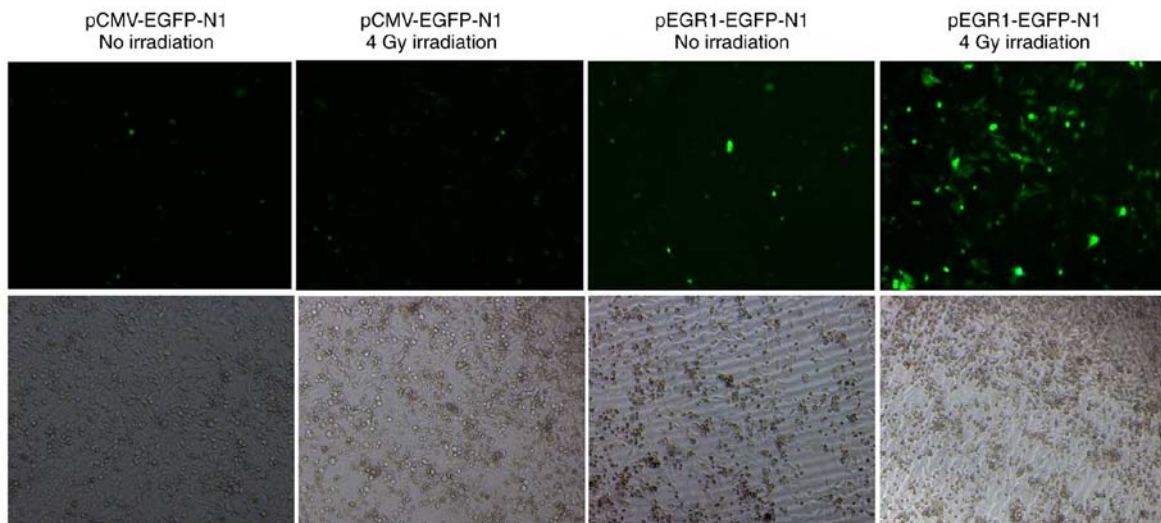


Figure 1. Irradiation-activated EGR1 promoter drives EGFP expression in transiently transfected HeLa cells. HeLa cells were transiently transfected with pEGFP-N1 or pEGR1-EGFP-N1 and exposed to irradiation of 4-Gy X-rays. In the upper panels, green fluorescence protein was observed, and its relative optical microscopy images were shown in the lower panels. Particularly, increased EGFP was visualized when the EGR1 promoter was exposed to X-rays (upper right panels). Magnification, x100. EGFP, enhanced green fluorescent protein; EGR1, early growth response factor-1.

com/products/secondary-antibodies/anti-rabbit-igg-hrp-linked-antibody/7074?Ntk=Products&Ntt=7074) for 1.5 h at room temperature. The immunolabeled proteins were detected using an immobilon western chemiluminescent HRP Substrate (WBKLS0500; MilliporeSigma™).

Anchorage-independent growth analysis. HeLa cells infected with LV-mCherry(2A)puro-hING4 or pEGR1-LV-mCherry(2A)puro-hING4 were trypsinized and suspended in culture medium. A total of 900 cells were seeded in each well of a 6-well plate with complete medium and were grown for 24 h. The cells were then radiated by X-rays at a dose of 8, 6, 4, 2 and 0 Gy. When visible clones were observed for cells free from irradiation, all treated cells were subjected to fixation by 100% methanol at 4°C for 10 min and then stained for 1 h with 0.1% crystal violet at room temperature. After removing the dye, colonies containing >50 cells were counted (captured using a digital camera and the colonies in the photos were counted).

Cobalt chloride-imitated hypoxia. Cobalt chloride is an additive and widely used to imitate hypoxia in cell culture (20). The present study used cobalt chloride (cat. no. 232696; Sigma-Aldrich; Merck KGaA) at 100 μ M to treat the HeLa cells (at a density of 1.5×10^5 /ml), maintained at 37°C in a humidified atmosphere with 5% CO₂, following X-ray exposure.

Statistical analysis. Statistical analysis was performed using SPSS 20.0 software (IBM Corp). All experiments were performed in triplicate wells for each condition and repeated at least twice. Data are presented as the mean \pm standard error. Error bars indicate the standard deviation. Generally, the one-way ANOVA method was used to evaluate the differences among treatments. Multiple comparisons among the groups were performed using the Bonferroni method. P<0.05 was considered to indicate a statistically significant difference.

Results

Promoter of EGR1 is sensitive to X-ray irradiation. The pEGFP-N1 or pEGR1-EGFP-N1 plasmid, in which the CMV promoter was replaced by the promoter of the EGR1 gene, was transfected into HeLa cells. At 24 h after transfection, cells were subjected to irradiation with X-rays at 4 Gy and fluorescence was observed at 48 h post-exposure to X-rays. The results indicated that the CMV promoter did not respond to irradiation induction; however, the EGR1 promoter was sensitive to X-ray exposure (Figs. 1 and S1). Therefore, the EGR1 promoter was inducible by radiation and promoted the transcription and expression of its downstream gene.

HeLa cells stably integrated with the EGR1 promoter and the ING4 ORF cassette were screened and set up as a cell line. The cells expressed an intense red fluorescence protein, mCherry, following 4 Gy irradiation. By contrast, no red fluorescence was observed when the cells were not exposed to X-rays (Fig. 2A).

The present study further examined whether the EGR1 promoter was sensitive to X-ray irradiation based on translation levels. HeLa cells with an EGR1-driven ING4 ORF were exposed to irradiation at the 0, 2, 4, 6 or 8 Gy for 48 h, and proteins were then harvested and used to detect ING4 expression via western blot analysis. ING4 protein exhibited a dose-dependent expression pattern after the cells were irradiated with X-rays (Fig. 2C). However, HeLa cells integrated only with ING4 ORF did not exhibit enhanced ING4 protein expression following X-ray irradiation (Fig. 2B).

Therefore, it was concluded that the EGR1 promoter was sensitive to X-ray induction according to transient transfection-based green fluorescence protein, stably integration-based red fluorescence protein and ING4 protein expression. Since the purpose of the present study was to examine the responsiveness of the EGR1 promoter but not ING4 to irradiation, other negative controls, such as pEGR1-0, pCMV-ING4 and pING4 were not additionally evaluated.

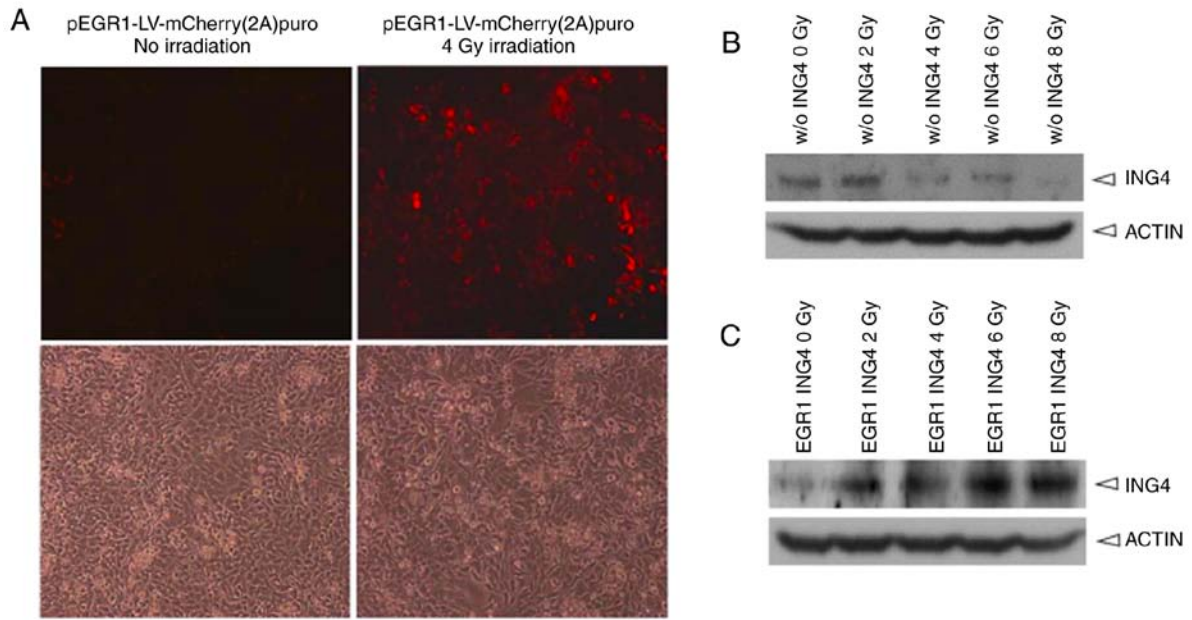


Figure 2. Irradiation-activated EGR1 promoter drives mCherry and ING4 expression in stably integrated HeLa cells. (A) HeLa cells were stably integrated with EGR1 promoter and mCherry ORF. mCherry expression increased due to the positively regulated promoter activity under irradiation (upper right panel). Magnification, x100. (B) ING4 expression was detected by western blotting. HeLa cells stably integrated with ING4 ORF without the EGR1 promoter irradiated by different doses of X-ray exhibited ING4 expression was hardly visible. (C) ING4 expression was monitored by western blotting. HeLa cells stably integrated with EGR1 promoter and ING4 ORF exhibited increased ING4 expression following treatment with a gradient of X-rays. EGR1, early growth response factor-1; ING4, inhibitor of growth 4; ORF, open reading frame.

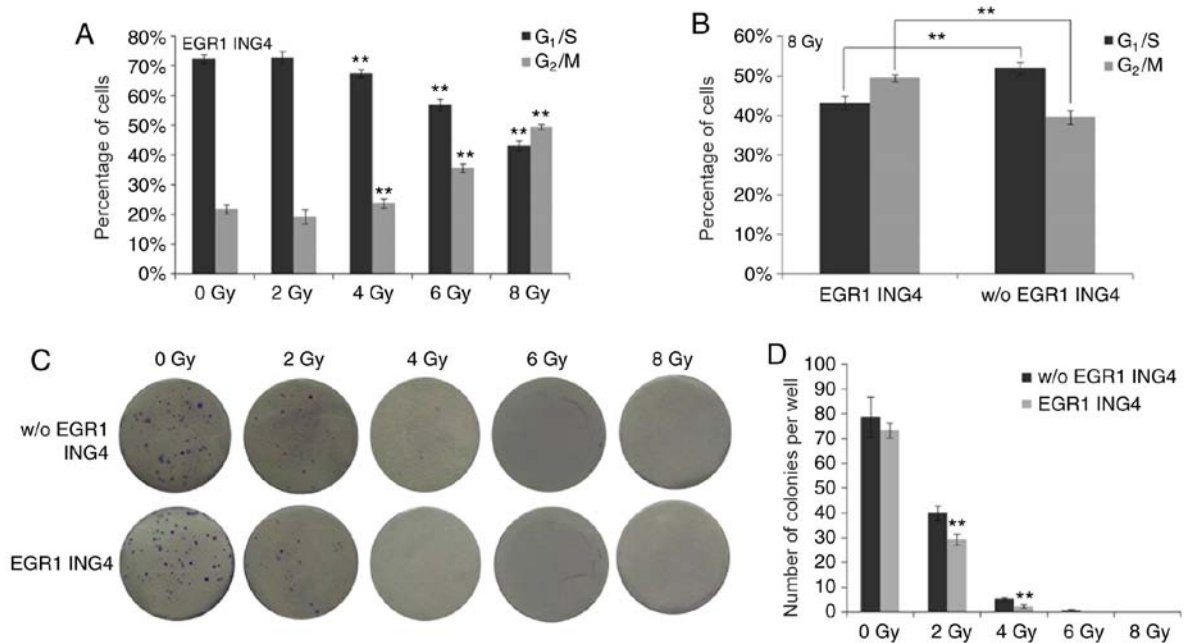


Figure 3. Cell cycle and proliferation of HeLa cells with irradiation-induced ING4 expression. (A) Percentage of G_1/S and G_2/M phase HeLa cells with stably integrated EGR1 promoter and ING4 open reading frame irradiated by X-rays. Compared with those of HeLa cells without irradiation, higher percentages of cells in G_2/M phase and lower percentages of cells in G_1/S phase were observed in HeLa cells treated with 4, 6 and 8 Gy irradiation. $^{**}P < 0.001$ vs. cell percentage without irradiation (0 Gy). (B) Distribution of phases of HeLa cells with or without inducible ING4 expression. ING4 expression significantly increased the percentage of cells in G_2/M phase and decreased the percentage of cells in G_1/S phase. $^{**}P = 0.008$ vs. without EGR1 promoter. (C) The photo of colony formation assay of HeLa cells with and without inducible ING4 expression under irradiation. With increasing irradiation, decreasing numbers of colonies were observed for both groups. (D) Comparison of the colony numbers between ING4-expressing HeLa cells and non-inducible cells. Once ING4 was induced, less colonies were observed in HeLa cells. $^{**}P < 0.001$ vs. cell number without irradiation (0 Gy). EGR1, early growth response factor-1; ING4, inhibitor of growth 4.

ING4 induces G_2/M phase arrest. With the increment of ING4 expression caused by the increasing doses of irradiation, the percentage of cells in the G_2/M phase gradually increased

along with a sequential decrease in the number of cells in the G_1/S phase ($P < 0.001$; Figs. 3A and 4). In particular, the percentage of cells in the G_2/M phase was statistically signifi-

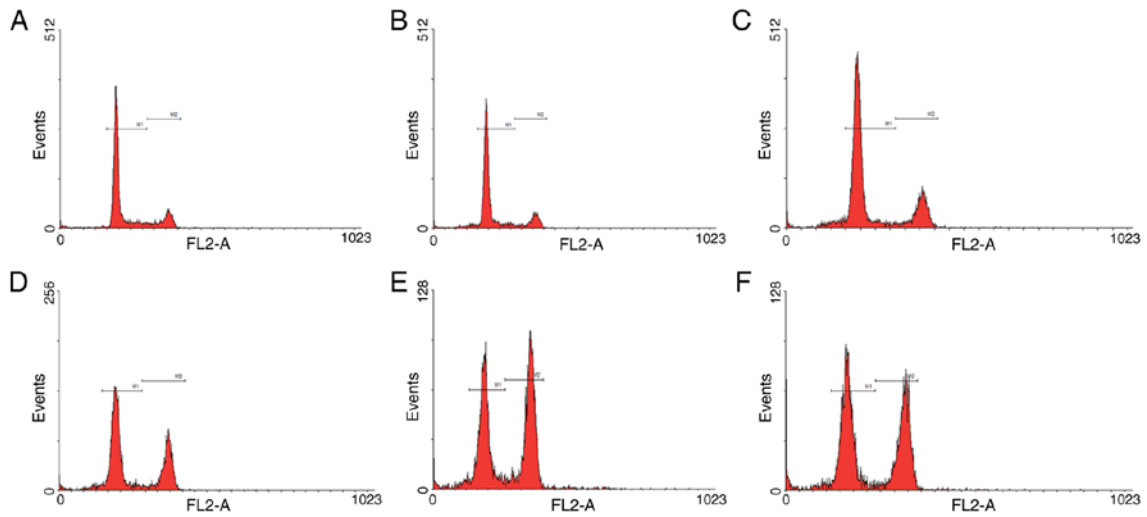


Figure 4. Cell cycle analyses of HeLa cells by flow cytometry under the control of different doses of irradiation. The cell counts of HeLa cells stably integrated with EGR1 promoter and ING4 ORF, irradiated by (A) 0, (B) 2, (C) 4, (D) 6 and (E) 8 Gy, in G₁/S and G₂/M phase. (F) The cell counts of HeLa cells stably integrated with ING4 ORF without irradiation-induced EGR1 promoter, and irradiated by 8 Gy, in G₁/S and G₂/M phase. Events, cell counts; M1, G₁/S phase; M2, G₂/M phase; EGR1, early growth response factor-1; ING4, inhibitor of growth 4; ORF, open reading frame.

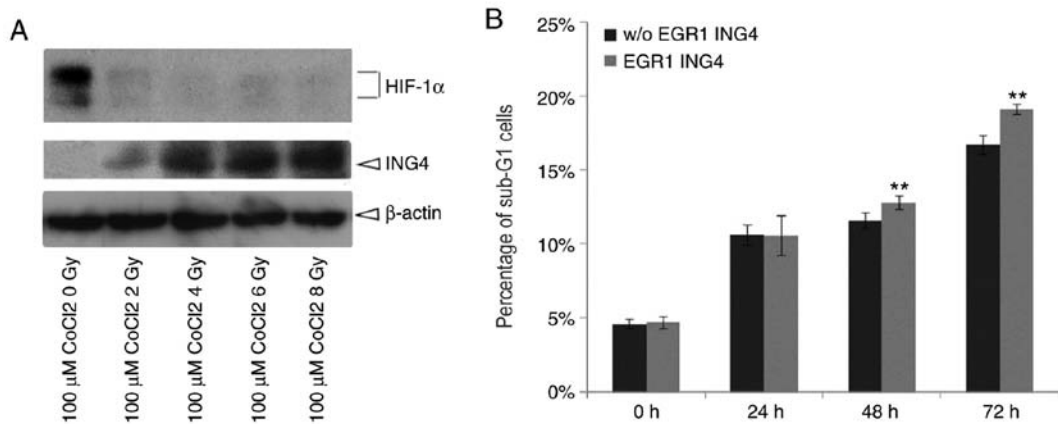


Figure 5. ING4 inhibits HIF-1 α expression and promotes cell apoptosis. (A) Inducible ING4 expression and its effect on HIF-1 α expression. When treated with CoCl₂-imitated hypoxia, HeLa cells exhibited high expression levels of HIF-1 α which were inhibited by ING4 induced by irradiation. (B) Effect of ING4 on HeLa cell apoptosis in CoCl₂-imitated hypoxia under 8 Gy irradiation. ING4 expression significantly promoted the apoptosis of HeLa cells at 48 and 72 h compared with cells transfected with 0-ING4 (without EGR1 promoter), **P<0.05.. CoCl₂, cobalt chloride; EGR1, early growth response factor-1; HIF-1 α , hypoxia-inducible factor 1 α ; ING4, inhibitor of growth 4.

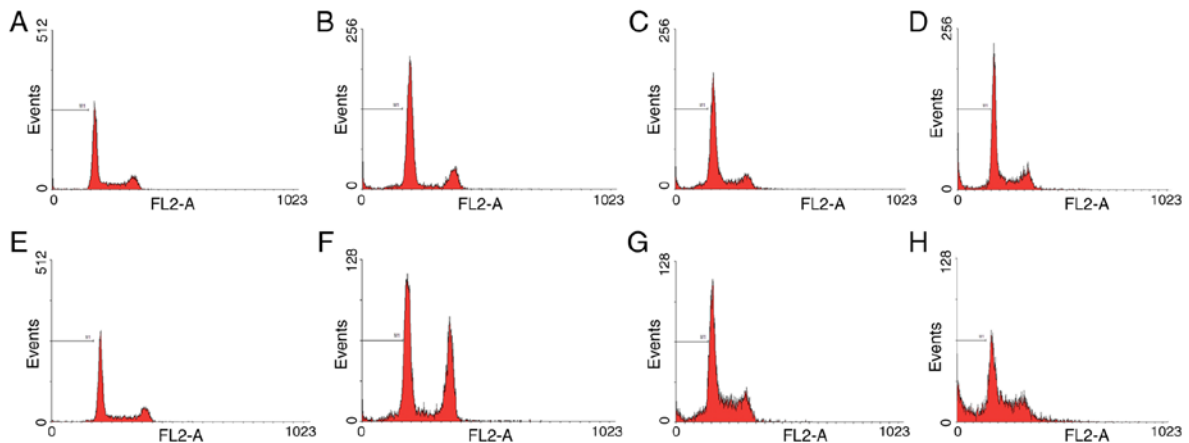


Figure 6. Apoptosis analysis of HeLa cells by flow cytometry with irradiation under hypoxia. Percentages of sub-G₁ phase HeLa cells stably integrated with ING4 ORF without irradiation-induced EGR1 promoter, irradiated and analyzed at (A) 0, (B) 24, (C) 48 and (D) 72 h post-irradiation. Percentages of sub-G₁ phase HeLa cells stably integrated with EGR1 promoter and ING4 ORF, irradiated and analyzed at (E) 0, (F) 24, (G) 48 and (H) 72 h post-irradiation. M1, percentages of sub-G₁ phase cells (apoptotic cells); EGR1, early growth response factor-1; ING4, inhibitor of growth 4; ORF, open reading frame.

cantly higher in the ING4-expressing HeLa cells than in the HeLa cells without ING4 expression when 8 Gy irradiation was employed ($P=0.005$). By contrast, the number of cells in the G₁/S phase was lower in the ING4-expressing HeLa cells than in the HeLa cells without ING4 expression ($P=0.008$; Figs. 3B and 4). Therefore, irradiation-induced ING4 expression led to G₂/M phase arrest, which may be responsible for the growth retardation (decreased number of the cells) of these HeLa cells (Fig. 3D).

ING4 inhibits the proliferation of HeLa cells. With the increase in the irradiation dose, ING4 expression was increased in HeLa cells, which resulted in less colonies being formed compared with in the HeLa cells not expressing ING4 (2 Gy, $P=0.009$; 4 Gy, $P=0.003$). By contrast, no statistically significant differences were observed in the number of colonies between the groups of HeLa cells not exposed to irradiation (0 Gy; Fig. 3C and D). No obvious colonies were observed following irradiation with 6 Gy or 8 Gy.

ING4 inhibits HIF-1 α expression. In the present study, following irradiation with X-rays at various doses, HeLa cells were treated with 100 μ M cobalt chloride for 24, 48 or 72 h. Proteins were then collected from HeLa cells. HIF-1 α expression markedly decreased while ING4 expression increased (Fig. 5A).

ING4 promotes irradiation-induced apoptosis under hypoxic conditions. Furthermore, the proportions of HeLa cells in the sub-G₁ phase under hypoxic conditions and following X-ray exposure were detected. At 48 and 72 h, ING4 increased the proportion of cells in the sub-G₁ phase following 8 Gy irradiation, which indicated that a greater number of HeLa cells was apoptotic ($P=0.04$ and $P=0.03$, respectively; Figs. 5B and 6). These results demonstrate that ING4 could promote cell apoptosis under hypoxic conditions.

Discussion

Hypoxic cancer cells are more resistant to irradiation compared with fully oxygenated cells (21). Therefore, the extent of tumor hypoxia is one of the most crucial biological factors affecting the outcomes of radiotherapy (22). Furthermore, HIF-1 has been identified to serve a pivotal role in hypoxia-mediated radioresistance (23). Research on the effects of hypoxia has increased in recent years; however, limited progress has been achieved in previous studies. On the other hand, dose escalation that aims to increase tumor control may observed the adverse effects of the normal tissues nearby. Therefore, enhancing the radiosensitivity of tumor cells, including overcoming hypoxia, is considered critical in order to achieve successful radiation therapy (24).

The strategies used to enhance radiosensitivity in the present study included three parts. At first, ING4 suppressed HIF-1 expression, which exerts a promoting effect on tumor growth under hypoxic conditions (21). Secondly, ING4 induced G₂/M phase arrest and the apoptosis of HeLa cells. Several previous studies have reported similar results (25-28). The third part is that a lentiviral gene therapy vector was constructed, containing human ING4 and its upstream promoter, EGR1, which share the radiation-inducible characteristics to activate

the transcription of downstream genes. When the experimental model was exposed to external irradiation, ING4 was activated by the EGR1 promoter and targeted to be expressed in irradiated sites. Therefore, the irradiation-sensitive promoter, EGR1, facilitated therapeutic gene expression under the control of ionizing radiation. Thus, the promoting effect on tumor growth by hypoxic cells was inhibited when HIF-1 was suppressed by ING4. Combined with the role of ING4 in regulating the cell cycle, synergetic radiosensitizing effects were achieved simultaneously with radiation therapy. The findings of the present study may provide novel strategies for the application and efficacy evaluation of radiosensitization in cervical cancer.

Increasing evidence has indicated that ING4 serves an important role in cancer progression as a tumor suppressor (5). In lung cancer tissues, decreased ING4 expression is present in ~50% of cases and is associated with lymph node metastasis (29). Together with the results of other surveys, ING4 is a promising target in gene-radiotherapy (30-32).

Radiotherapy combined with tumor suppressor genes is increasingly being used in tumor therapy (5). To achieve combined therapy, the genetic modification of tumor cells is critical. In the present study, ING4, as a tumor suppressor, was introduced into HeLa cells by lentiviral transfection under the control of the EGR1 promoter. The EGR1 promoter is sensitive to radiation. Therefore, the combination of ING4 and radiation was more effective in the cellular model of human cervical cancer treatment. The findings of the present study may provide promising strategies for use in the treatment of other types of cancer. However, these findings warrant further investigation using *in vivo* models.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

HPX and YNJ were involved in design of the work. TM was involved in acquisition of data and drafting the initial manuscript. HPX, XW and YNJ were the major contributors in revising the manuscript for important intellectual content. TM and HPX gave final approval of the version to be published. TM, XW, RG, WTS and MZ performed experimental research and analyzed experimental data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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