# p53 activated by AND gate genetic circuit under radiation and hypoxia for targeted cancer gene therapy

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Radio-activated gene therapy has been developed as a novel therapeutic strategy against cancer; however, expression of therapeutic gene in peritumoral tissues will result in unacceptable toxicity to normal cells. To restrict gene expression in targeted tumor mass, we used hypoxia and radiation tolerance features of tumor cells to develop a synthetic AND gate genetic circuit through connecting radiation sensitivity promoter $cArG_6$, heat shock response elements SNF1, HSF1 and $HSE_4$ with retroviral vector plxsn. Their construction and dynamic activity process were identified through downstream enhanced green fluorescent protein and wtp53 expression in non-small cell lung cancer A549 cells and in a nude mice model. The result showed that AND gate genetic circuit could be activated by lower required radiation dose (6 Gy) and after activated, AND gate could induce significant apoptosis effects and growth inhibition of cancer cells *in vitro* and *in vivo*. The radiation- and hypoxia-activated AND gate genetic circuit, which could lead to more powerful target tumoricidal activity represented a promising strategy for both targeted and effective gene therapy of human lung adenocarcinoma and low dose activation character of the AND gate genetic circuit implied that this model could be further exploited to decrease side-effects of clinical radiation therapy.

As a novel cancer treatment, radio-activated gene therapy, in which an ionizing radiation-inducible regulatory sequence is linked with tumor-therapeutic genes for the purposes of dual lethal effects by both radiation and the radiation-inducible gene, has attracted considerable interest in recent years.[1] Egr-1 gene promoter, which is a member of the radiation responsive gene family and consists of 10 bp motifs known as cArG [CC(A/T)6GG] DNA elements have been identified to control radiation response after being activated by reactive oxygen species produced on exposure to ionizing radiation (IR), and thereafter has widely been used as an ionizing radiation-inducible sequence in radio-genetic therapy.[2–5] This IR-stimuli response exhibits an apparent advantage of overcoming low target expression by selectively controlling therapeutic gene expression and produces ideal antitumor effects, especially in inoperable malignancy with high radiation sensitivity, such as nasopharyngeal carcinoma. However, one challenge of this strategy is peritumoral normal tissues in the radiation field can inevitably receive unwanted radiation and the expression of therapeutic gene in these tissues will apparently result in an unacceptable degree of toxicity to normal cells.[6,7] Another problem is that most radio-activated promoters usually respond to a single fraction dose of 20–50 Gy, which is far higher than the clinical conventional dose of

2 Gy.[8] Therefore, if the therapeutic gene can be regulated by low doses of radiation to express within the tumor tissues, marked antitumor effects and the protection of normal tissues will be accomplished.

The hypoxic microenvironment within numerous types of solid tumors, has been identified as one of the main reasons for radiotherapy and chemotherapy refractory of malignancy.[9,10] Thus this unique hypoxia character can be exploited for selective cancer treatment. Many investigators have reported the potential exploitation of tumor-specific microenvironment-hypoxia for the targeted expression of therapeutic genes to kill cancer cells.[9–12] In order to combine radiation-mediated gene therapy with hypoxia and to maximize the activation of the therapeutic genes within the tumor mass, we propose to devise a special vector, which can be activated by radiation and hypoxia together but not either alone to control the therapeutic gene expression within hypoxic tumor cells in radiation field instead of peritumoral or other hypoxic normal tissues. By restraining therapeutic gene expression within tumors, this strategy can effectively accomplish targeted treatment, while avoiding toxicity to normal tissues. And this regulation modality is called AND gate gene circuit. AND gate means when all upstream promoters are activated, the downstream elements will be transcribed and off otherwise.[13] In

our experiments, by applying DNA recombination technology, we constructed an AND gate genetic circuit based on heat shock response elements. The heat shock response is also called "stress response". It means when cells receive heat shock stimulus, sucrose non-fermenting 1 (SNF1) protein kinase in cytoplasm is activated first and phosphorylates heat shock factor-1 (HSF1), upon stimuli such as ethanol, injury or hypoxia received, phosphorylated HSF1 will combine to heat shock element (HSE) and induces a series of downstream gene expression.[14,15] Hence, in our study, we try to combine hypoxia, which occurs in solid tumor mass and can activate heat shock response with IR as the heat shock stimuli to construct an AND gate genetic circuit (Fig. 1a) and test its activity and targeted antitumor ability in vitro and in vivo. In this AND gate, after being stimulated by radiation, radiosensitivity promoter $cArG_6$ activates SNF1 expression and phosphorylates HSF1 follows, meanwhile hypoxia induces the combination of HSF1 with HSE, and the downstream therapeutic gene would be transcribed and accomplish target expression (Fig. 1a).

Although application of radiation treatment with hypoxia for cancer gene therapy has been reported elsewhere;[3,10,16] for example, Greco et al.[10,16] constructed hypoxia- and radiation-activated Cre/loxP 'molecular switch' vectors, which include Egr1-hypoxia response element (HRE) dual-sensitive promoters to drive the herpes simplex virus thymidine kinase (HSV-TK) suicide gene expression, and through transfection into human breast and glioma cells, these vectors were identified as being more efficient than using Egr1 or HRE promoter separately under radiation and hypoxia. However, these strategies that can be activated by radiation, hypoxia or combined stimuli exhibit low tumor-targeting properties because some

normal cells are also in hypoxic environments and can get the lethal effects by suicide gene expression, such as the bone marrow stem cells.[17]

In the present study, we used radiotherapy and hypoxia-mediated gene therapy to construct an AND gate genetic circuit through combination of the heat shock elements with the radiation response promoter $cArG_6$ and tested whether the constructed AND gate could be activated by radiation and hypoxia stimuli. In vitro and in vivo experiments which represented the therapeutic response to non-small-cell lung carcinoma (NSCLC) were conducted to analyze the targeted expression of exogenous gene and antitumor effects, anti-oncogene wild type p53 (wtp53) was adopted as a model of suicide gene. Our results indicated that the AND gate genetic circuit could be activated by radiation and hypoxia stimuli together instead of alone and exhibited targeted expression. The activated AND gate vector further showed significant effects of growth inhibition and apoptosis promotion in vitro and in vivo. And through utilization of radiation promoter, decreased radiation doses as the stimulus could be achieved and reduced damage of peritumoral normal tissues might be accomplished. These findings provide support for a novel targeted approach through combination radiation treatment with hypoxia under the temporal and spatial control of gene therapy for NSCLC. The characteristic of low radiation dose activity implies that this AND gate can be exploited as the targeted sensitization strategy in radiation therapy.

## Materials and Methods

**Plasmids and reagents.** The plxsn-enhanced green fluorescent protein (EGFP) and plxsn-wtp53 vectors were constructed via a multi-stage process as Figure 1b. The self-inactivating (SIN) retroviral empty vector plxsn carrying EGFP was used as the framework. The SIN vector was generated by deletion of a 70 bp AscI–PshAI fragment within the 5′-long terminal repeats (LTR) of plxsn. $cArG_6$ promoter and $HSE_4$ reporter were synthesized by utilizing oligodeoxyribonucleotides reannealing technology (Sangon Company, Shanghai, China) which could retain native activity of the constructed sequences, $cArG_6$ gene was subcloned into the SIN site as transcriptional control subsequences in 5′-LTR sequence of plxsn with AscI–PshAI enzyme sites, $HSE_4$ gene was subcloned into downstream gag site with HpaI and XhoI enzyme sites. SNF1, HSF1 and wtp53 cDNA sequences were generated by proofreading polymerase chain reaction (PCR) (Table 1) from puc18, pOTB7 and pCMV6-XL4 plasmids, respectively, and verified by sequencing. 1902 bp SNF1 gene ligated into the BsrgI site was inserted into downstream of $cArG_6$ and 1600 bp HSF1 gene ligated into EcoRI site were inserted into downstream of SNF1. Continuous intramolecular religation resulted in the reconstructed control vectors named plxsn-EGFP, in which SNF1 and HSF1 were driven only by the activity of the $cArG_6$ promoter. Therapeutic vectors named plxsn-wtp53 were constructed by replacing EGFP with wtp53 gene by XhoI and BamHI digestion. The plasmids DNA were purified using QIAGEN Plasmid Plus kit (Qiagen, Shanghai, China) and the assay was determined by Bradford methods (Beyotime, Jiangsu, China). All fragments of recombinant vectors were identified by 1% agarose gel electrophoresis stained with Gold View Nuclear Acid (Takara, Dalian, China) and compared sequences by BLAST. puc18 plasmids carrying SNF1, pOTB7 plasmids carrying HSF1 and pCMV6-XL4 plasmids carrying wtp53 were purchased from Promega (Madison, WI, USA). Enzymes
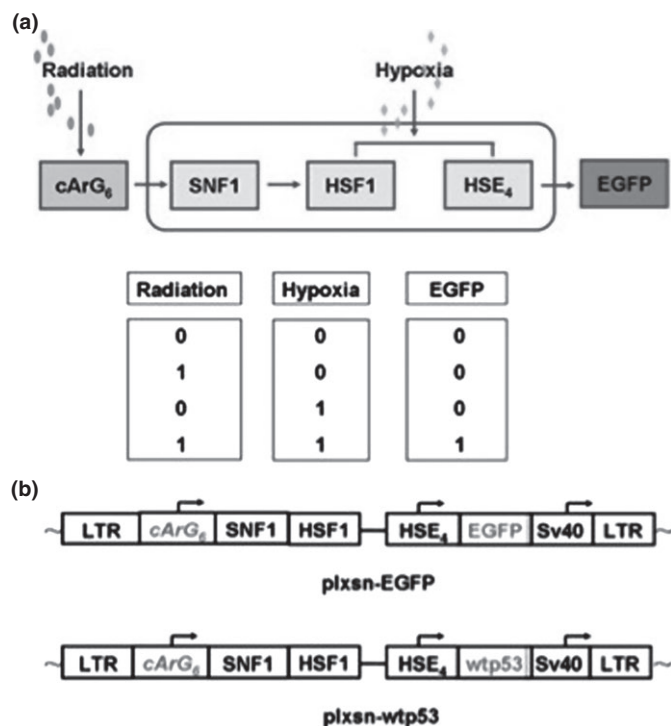
**Fig. 1.** AND gate genetic circuit. (a) AND gate based on heat shock response elements. In this AND gate, after stimulation by radiation, radiosensitivity promoter $cArG_6$ activated SNF1 and phosphorylated HSF1, meanwhile hypoxia induced combination of HSF1 with HSE and downstream gene EGFP would be transcribed. (b) Design of AND gate vectors. plxsn-EGFP: control vectors; plxsn-wtp53: therapeutic vectors.

**Table 1. Primer sequences of SNF1, HSF1 and wtp53**

| Primers | Nucleotide sequence | Expected size(bp) | Enzyme incision sites |
| --- | --- | --- | --- |
| SNF1 | | | |
| Forward | 5′-GAC<u>CTCGAG</u>ACCATGAGCAGTAACAACAACACAAACACAGCAC-3′ | 1902 | *Bsr*gI and *Eco*RI |
| Reverse | 5′-GAC<u>ATCGAT</u>ATTGCTTTGACTGTTAACGGCTAATTC-3′ | | |
| HSF1 | | | |
| Forward | 5′-<u>AAAACCGGT</u>ATGGATCTGCCCGTGGGCCC-3′ | 1600 | *Eco*RI and *Hpa*I |
| Reverse | 5′-GAC<u>GCGGCCGC</u>GGAGACAGTGGGGTCCTTGGC-3′ | | |
| wtp53 | | | |
| Forward | 5′-GAC<u>GAATTC</u>ATGGAGGAGCCGCAGTCAGAT-3′ | 1200 | *Xho*I and *Bam*HI |
| Reverse | 5′-GAC<u>CTCGAG</u>TTAGTCTGAGTCAGGCCCTTCTGT-3′ | | |

Enzyme incision sites were designed as the underlined sequences.

(MBI Fermentas, Vilnius, Lithuania) were used according to the manufacturers' instructions. The synthetic oligonucleotides sequences were: cArG$_6$ 5′-GATCT(CCTTATTTGG)$_6$GCGCT-3′, 5′-CGC(CCAAATAAGG)$_6$A-3′; HSE$_4$ 5′-GATCT(TTCTA-GAAAGTTC)$_4$GCGCT-3′, 5′-CGC(GAACTTTCTGAA)$_4$A-3′.

**Cell lines and cell culture.** A549 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained in RPMI 1640 culture medium containing 10% heat inactivated fetal bovine serum (FBS; Gibco, Grand Island, NY, USA), 1% penicillin-streptomycin (Sigma, St. Louis, MO, USA) and 2 mM L-glutamine (Sigma). All of the cells were incubated in a humidified incubator at 37°C with 5% $CO_2$, 95% air and tested free of mycoplasma infection.

**Transient transfection and radiation-hypoxia intervention.** DNA transient transfection experiments were used according to Lipofectamine 2000s instructions (Invitrogen, Carlsbad, CA, USA). For experiments under radiation and hypoxia conditions, after 24 h of transfection, cells in the radiation group were exposed to 6 Gy[18] using a Pantak PCM 1000 X-ray generator (Pantak, East Haven, CT, USA) (200 kV, 15 mA, filter 0.5 mm aluminum and 0.5 mm cuprum, 0.47 Gy/min); cells in the hypoxia group were placed in a 37°C hypoxia incubator with 1% $O_2$, 94% $N_2$ and 5% $CO_2$ for 3 h;[19] cells in the radiation and hypoxia group were irradiated by 6 Gy and then placed into a hypoxic incubator for 3 h, and finally, all cells were returned to 37°C normal homoeothermic incubator to culture overnight. After 24, 48 and 72 h, cells were collected by using trypsin (0.25% trypsin, 1 mM EDTA; Invitrogen) and counted for the following experiments.

**RT-PCR analysis.** RNA extraction was performed according to the Trizol protocol (Invitrogen) and cDNA was generated using the M-MuLV reverse transcription system (Fermentas). One microliter of cDNA samples was subjected to PCR analysis. PCR reaction conditions were as follows: initial denaturation at 94°C for 1 min and 32 cycles of amplification (94°C for 1 min, 52.5°C for 1 min, and 72°C for 2 min), followed by a final extension step for 10 min at 72°C. The primers were used as follows: GAPDH forward: 5′-ACCACAGTCCATGC-CATCAC-3′; reverse: 5′-TCCACCACCCTGTTGCTGTA-3′, 491 bp; wtp53 forward: 5′-GACGAATTCATGGAGGAGCCG-CAGTCAGAT-3′; reverse: 5′-GACCTCGAGTTAGTCTGAGT CAGGCCCTTCTGT-3′, 1200 bp.

**Western blotting analysis.** Whole proteins of exponentially growing cells or tissues were collected in lysis buffer (Promega) containing the complete cocktail of protease inhibitors (Roche, Branchburg, NJ, USA). The protein concentration of the cell lysates was determined by Bradford methods (Bey-

otime) and then was regulated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. Equal amounts of total proteins were loaded to 12% polyacrylamide gels and electrophoresed to separate proteins of interest at 120 V for 2 h, then the proteins were transferred to PVDF membranes (Millipore Corporation, Bedford, MA, USA) using a semi-dry method (Bio-Rad, Hercules, CA, USA). The membranes were blocked and incubated with primary antibodies overnight at 4°C and secondary antibodies for 1 h at 37°C. At last, targeted proteins were detected through the ECL protocol (Beyotime), scanned and analyzed by automatic image analyzer. β-actin was used as a control and obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All of the primary antibodies and dilutions contained the following: donkey anti-SNF1 antibody (1:2000; Santa Cruz), mouse anti-HSF1 antibody (1:1000; Santa Cruz), rabbit anti-p53 antibody (1:500; Cell Signalling, Danvers, MA, USA), and rabbit anti-EGFP antibody (1:500; Beyotime). Secondary antibodies coupled to horseradish peroxidase (HRP) were purchased from Beyotime.

**Cell cycle analysis.** At least $5 \times 10^6$ cells per milliliter were collected and fixed with 70% ethanol at 4°C overnight, then incubated with RNase (Sigma) and resuspended in propidium iodide (PI) staining solution for 1 h, at last separated by using the FACS Vantage SE (Becton Dickinson, Heidelberg, Germany).

**Apoptosis analysis.** Cells were harvested, washed twice with PBS, and resuspended in $1 \times$ binding buffer at the concentration of $1 \times 10^6$ cells per milliliter. 100 μL of solution was mixed with 5 μL Annexin V-FITC and PI for 15 min, and then 400 μL $1 \times$ binding buffer was added. At last, samples were carried out to analysis by using a FC500 flow cytometer (Becton Dickinson) and the percentage of apoptotic cells was assessed by CXP software (Becton Dickinson).

**Cell proliferation assay.** Cell proliferation assay was performed with the Cell Counting Kit-8 (CCK-8; Dojindo, Tokyo, Japan). A549 cells transfected with plxsn-wtp53 vectors received 6 Gy of radiation and hypoxia treatments and then were seeded in 96-well plates at 2000 cells/well. At 0, 24, 48 and 72 h, 10 μL CCK-8 reagent was added to each well and incubated the plate for 2 h at 37°C. By using a microplate reader (Bio-Rad), the absorbance (450 nm) of cells was measured in triplicate and the data were collected.

**Animal experiments.** Athymic BALB/c nude mice, male, 5 weeks old, were maintained under specific pathogen-free conditions in Experimental Animal Center of Third Military Medical University. Mice were manipulated according to protocols approved by Chongqing Medical Experimental Animal Care Commission. A549 cells were harvested and

resuspended in PBS at a concentration of $5 \times 10^7$ cells per milliliter, then injected with 200 µL/spot into two flank sides of the mice subcutaneously. Three to five weeks later, when bearing tumor volume reached 60 mm$^3$, mice in vector injection group were treated to inject the mixture (20 µL) of 0.8 µg of the plxsn-EGFP or plxsn-wtp53 plasmids and 19.2 µL of Lipofectamine 2000 for each mouse intratumorally (days 1, 3, 5); mice in radiation group were irradiated (2 Gy) by focusing the xenografted tumors with Pantak PCM 1000 X-ray generator (200 kV, 15 mA, filter 0.5 mm aluminum, 1.41 Gy/min; Pantak) on days 2, 4, 6; mice in the combined treatment group were performed plasmids injection on days 1, 3, 5, and then the local radiation on days 2, 4, 6. All of the mice ($n$ = 10 mice per group) were monitored by measuring tumor growth and ordinary circumstances every 2 days, the tumor volume = length × width$^2$/2. Half of the animals were killed on day 10 after treatments, xenografts and peritumoral tissues were removed separately and fixed with 4% paraformaldehyde for the next step experiments.

**Histologic analysis.** Fixed tumors were paraffin-embedded, sectioned, stained with Haematoxylin Eosin (H&E) and examined by the light microscope.

As for the detection of apoptosis *in situ* in xenografts, terminal deoxynucleotidyl transferase-meditaed dUTP nickend labeling (TUNEL) assay to detect fragmented DNA *in situ* was carried out using the In Situ Cell Death Detection Kit (Roche). Briefly, sections were quenched in 2% hydrogen peroxide. The optimal dilution and incubation with the TdT enzyme was 1:54 for 1.5 h at 37°C. Then, the antidigoxigenin antibody (1 : 1000; 1 h at room temperature; Roche) was incubated. The reaction was visualized using diaminobenzidine tetrahydrochloride (Roche) as the chromogen, followed by a methyl green counterstain. The number of positive cells was determined by light microscopy at ×400 magnification.

**Immunohistochemical (IHC) analysis for detection of wtp53 and HIF-1α expression in xenografts.** Excised tumors were fixed in paraffin. Slices (5 µm) were deparaffinized with Xylene and rehydrated with a series of alcohol and water mixtures and finally with water. To quench endogenous peroxidase, the tissue sections were exposed for 30 min to 0.3% hydrogen peroxide ($H_2O_2$)-methanol solution. Microwave heating (4 min 500 W, 20 min defrost and 15 min room temperature) was used in the presence of Tris-EDTA buffer (0.01 M, pH 9.0) to achieve antigen retrieval prior to application of the primary monoclonal antibody. PBS (0.1 M, pH 7.3) plus Tween-20 (0.1%) were used to wash slides between the two steps. Then the sections were blocked by 5% normal goat serum (NGS) for 30 min in a moist chamber at 37°C, incubated with primary antibodies (p53: 1:250; Cell Signalling; HIF-1α: 1:200; Milipore Corporation) overnight at 4°C and secondary antibodies for 30 min at 25°C. Bound peroxidase was developed using 0.033% hydrogen peroxide in 10% diaminobenzidine (DAB; Dako, Glostrup, Denmark) for 7 min. After washing in distilled water, the sections were counterstained with hematoxylin for 1 min, dehydrated and mounted. Immunostained sections of the selected tumors were viewed by means of a Zeiss Axioskop 40 FL microscope (Carl Zeiss, Thornwood, NY, USA).

**Statistical analysis.** The data were presented as mean ± SD. Statistical analysis was performed using the SPSS 13.0 software (spss Inc. chicago IL). Differences between groups were compared by the ANOVA test. In all assays, the probability value ($P$) of <0.05 was considered statistically significant. Error bars represent SD of three independent experiments.

## Results

**Specific and enhanced activation of the synthetic AND gate genetic circuit in human A549 cells and in tumor-bearing athymic mice.** To obtain high-extension expression levels of radiation inducible transcription activity, we prepared a synthetic 70 bp cArG$_6$ sequence and subcloned into the transcription initiation sites (tcs1) of 5'-LTR sequences. Construction of AND gate vectors was achieved by conjugation SNF1, HSF1, HSE$_4$ and wtp53 into the downstream of cArG$_6$ promoter in turn. After gene recombination, two plasmids were formed: control vector named plxsn-EGFP and therapeutic vector named plxsn-wtp53 (Fig. 1b). To assess whether the AND gate mechanism could function after post-radiation treatment and under hypoxia, by transient transfection assay, changes in gene expression were observed in A549 cells. As shown in Figure 2a, cells transfected with plxsn-EGFP expressed apparent EGFP after exposure to radiation (6 Gy) and hypoxia (1%, 3 h) instead of other cells receiving radiation or hypoxia treatment alone. And after activation, EGFP protein expression changed in a time-dependent manner with the lowest level detection at 24 h and the highest level at 72 h. The result demonstrated that our genetic circuit possessed AND gate character, which could only be activated by radiation and hypoxia and further indicated that the cArG$_6$ promoter was silent without radiation stimulation.

The activation process of the AND gate genetic circuit was further confirmed through detection of downstream gene expression in A549 cells. Based on EGFP expression, we chose 48 h as the proper time when downstream protein expression was higher. As Figure 2b,c shown, when the levels of protein expression were standardized by β-actin expression, A549 cells transfected by plxsn-wtp53 and treated with radiation and hypoxia expressed higher SNF1 proteins compared with transfected cells with exposure of radiation or hypoxia ($P$ < 0.01). HSF1 and wtp53 protein induction expressed similar phenomenon that was the highest level in transfected cells with both stimuli. The western-blotting analysis revealed that radiation response promoter cArG$_6$ in the constructed vector kept their activity of inducing downstream gene expression under radiation stimulus. Furthermore, upregulated SNF1, HSF1 and wtp53 expression demonstrated that our synthetic AND gate vectors could be activated by combined treatment of radiation and hypoxia.

We then examined the engineering of the components of the AND gate vector in A549 cells. Cells were transiently transfected with plxsn, plxsn-EGFP (lacking SNF1 element) or plxsn-wtp53 (lacking SNF1 element), plxsn-EGFP (lacking HSF1 element) or plxsn-wtp53 (lacking HSF1 element), plxsn-EGFP (lacking HSE$_4$ element) or plxsn-wtp53 (lacking HSE$_4$ element), plxsn-EGFP or plxsn-wtp53 vectors and then received 6 Gy of radiation and hypoxia treatments. After 48 h, downstream gene expression was measured. As Figure 3a shown, after promoter activated, EGFP protein was observed to only express in cells transfected with plxsn-EGFP and received radiation and hypoxia treatments, and lacking any elements, the EGFP protein could not be observed. As for wtp53 expression, similar results (the highest level of wtp53 mRNA and protein expressed in cells transfected with plxsn-wtp53 under exposure of radiation and hypoxia) could be detected (Fig. 3b,c). The data told us that in the AND gate genetic
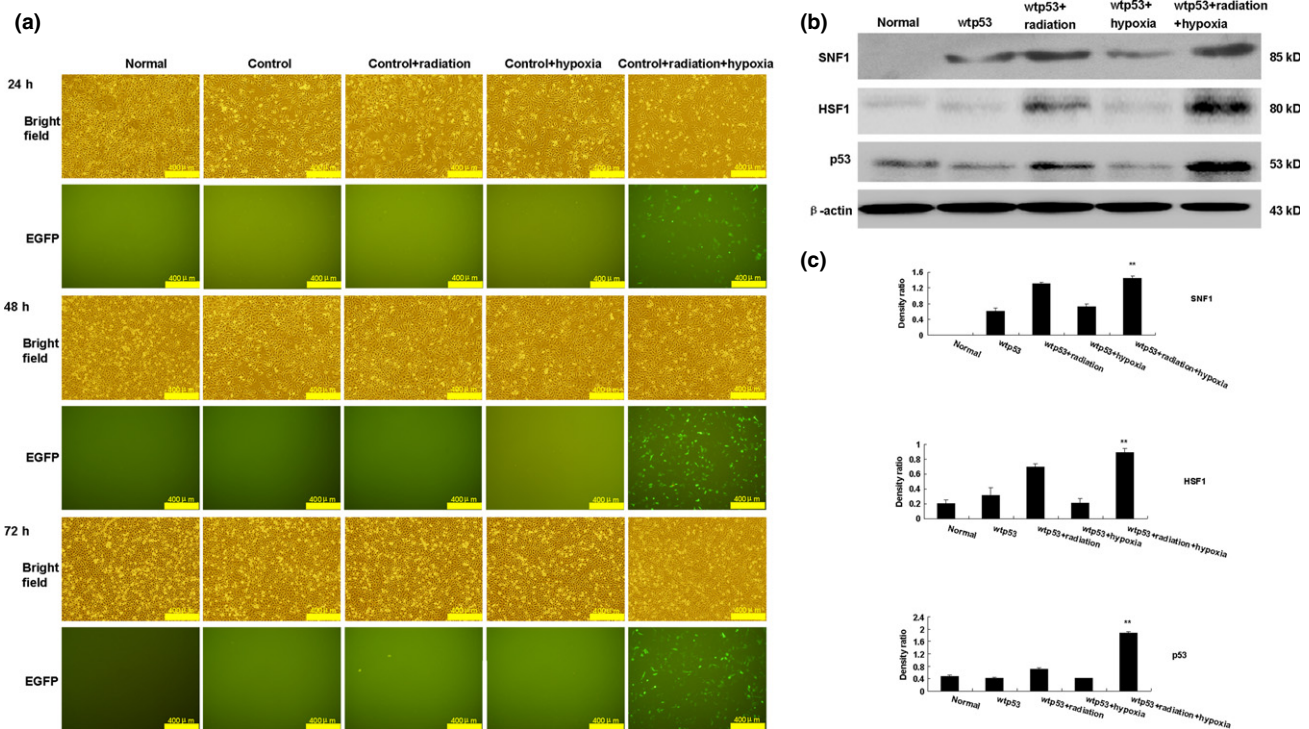
**Fig. 2.** Identification of synthetic AND gate activation through EGFP, SNF1, HSF1 and wtp53 expression in A549 cells. (a) EGFP expression in A549 cells treated for 24, 48 and 72 h. Cells in bright fields were negative control. (b) SNF1, HSF1 and wtp53 protein expression was detected by western blotting at 48 h in A549 cells receiving different treatments. β-actin was a control. (c) Quantitative analysis of SNF1, HSF1 and wtp53 proteins ($n = 3$ for each group, with the experiments carried out in triplicate). Data were shown as the means ± SD from three independent experiments. ** wtp53 + radiation + hypoxia group compared to all the control groups: $P < 0.01$. Normal: A549 cells without treatment; Control: A549 cells transiently transfected with plxsn-EGFP plasmids; Control + radiation: A549 cells transiently transfected with plxsn-EGFP plasmids and receiving 6 Gy of radiation treatment; Control + hypoxia: A549 cells transiently transfected with plxsn-EGFP plasmids and receiving 1% $O_2$ treatment; Control + radiation + hypoxia: A549 cells transiently transfected with plxsn-EGFP plasmids and receiving 6 Gy of radiation and 1% $O_2$ treatments; wtp53: A549 cells transiently transfected with plxsn-wtp53 plasmids; wtp53 + radiation: A549 cells transiently transfected with plxsn-wtp53 plasmids and receiving 6 Gy of radiation treatment; wtp53 + hypoxia: A549 cells transiently transfected with plxsn-wtp53 plasmids and receiving 1% $O_2$ treatment; wtp53 + radiation + hypoxia: A549 cells transiently transfected with plxsn-wtp53 plasmids and receiving 6 Gy of radiation and 1% $O_2$ treatments.

circuit, every element was necessary and could be activated in turn after exposure to radiation and hypoxia stimuli.

To further identify whether the constructed AND gate genetic circuit could be used for *in vivo* experiments, the HIF-1α expression was detected in A549 xenografts. In the present study, HIF-1α (endogenous hypoxia marker[20]) was detected in all A549 xenografts regardless of whether patients were receiving treatments or not and there was no significant difference in the amount and specificity in different groups (Fig. 4a, b). The result indicated that the tumor xenografts were exposed to hypoxia environment *in vivo*; therefore, analysis of enhanced and lengthened transcriptional activity of the AND gate genetic circuit in athymic mice was feasible. EGFP and wtp53 expression in peritumoral normal tissues and xenografts receiving plxsn-EGFP or plxsn-wtp53 injection under 6 Gy of radiation were further checked by western blotting. As expected, significant differences in different tissue and different groups of the two genes were observed. EGFP was not detected in peritumoral normal tissues, and in tumor tissues, the level of EGFP protein was significantly upregulated, especially in the central parts of xenografts ($P < 0.01$) (Figs 4c and 6d). Similar results were also found in wtp53 expression. These results indicated that activated AND gate genetic circuit induced enhanced expression of downstream anti-oncogenes in solid tumors and these findings were consistent with researches *in vitro*.

**Enhanced tumoricidal activity of activated AND gate genetic circuit *in vitro* and *in vivo*.** The antitumor ability of the AND gate genetic circuit *in vitro* was tested through examination of cell cycle, apoptosis and proliferation rates in A549 cells. As Figure 5a–d shows, in A549 cells owning activated AND gate, evident G1–G2 phase arrest was observed at 24 h ($P < 0.05$), 48 h ($P < 0.05$) and 72 h ($P < 0.05$). As for apoptosis detection, 26% of average apoptosis rate at 24 h, 68% at 48 h and 60% at 72 h were measured in the transfected plxsn-wtp53 cells exposed to radiation and hypoxia, and the rates were much higher ($P < 0.01$) than other groups with no AND gate activation (Fig. 5e–h). Moreover, proliferation inhibition of A549 cells with activated AND gate at 48 h ($P < 0.05$) and 72 h ($P < 0.05$) (Fig. 5i) was observed. All of the above data strongly implied that activated AND gate was able to induce a marked and enhanced cytotoxicity to NSCLC cells, such as the mitosis arrest, apoptosis promotion and proliferation progression inhibition. Additionally, we also could see that radiation caused minor antitumor effects *in vitro*, and the result was consistent with previous studies.[21]

Enhanced treatment of the AND gate genetic circuit was further detected in A549 xenografts. As shown in Figure 6a, normal tumors grew steadily, doubled in size on Day 5 and exhibited 4-fold increase on Day 13. Similar growth patterns were observed in tumors treated with the plxsn-EGFP vectors injection (1.5-fold increase in mean tumor volume on Day 5
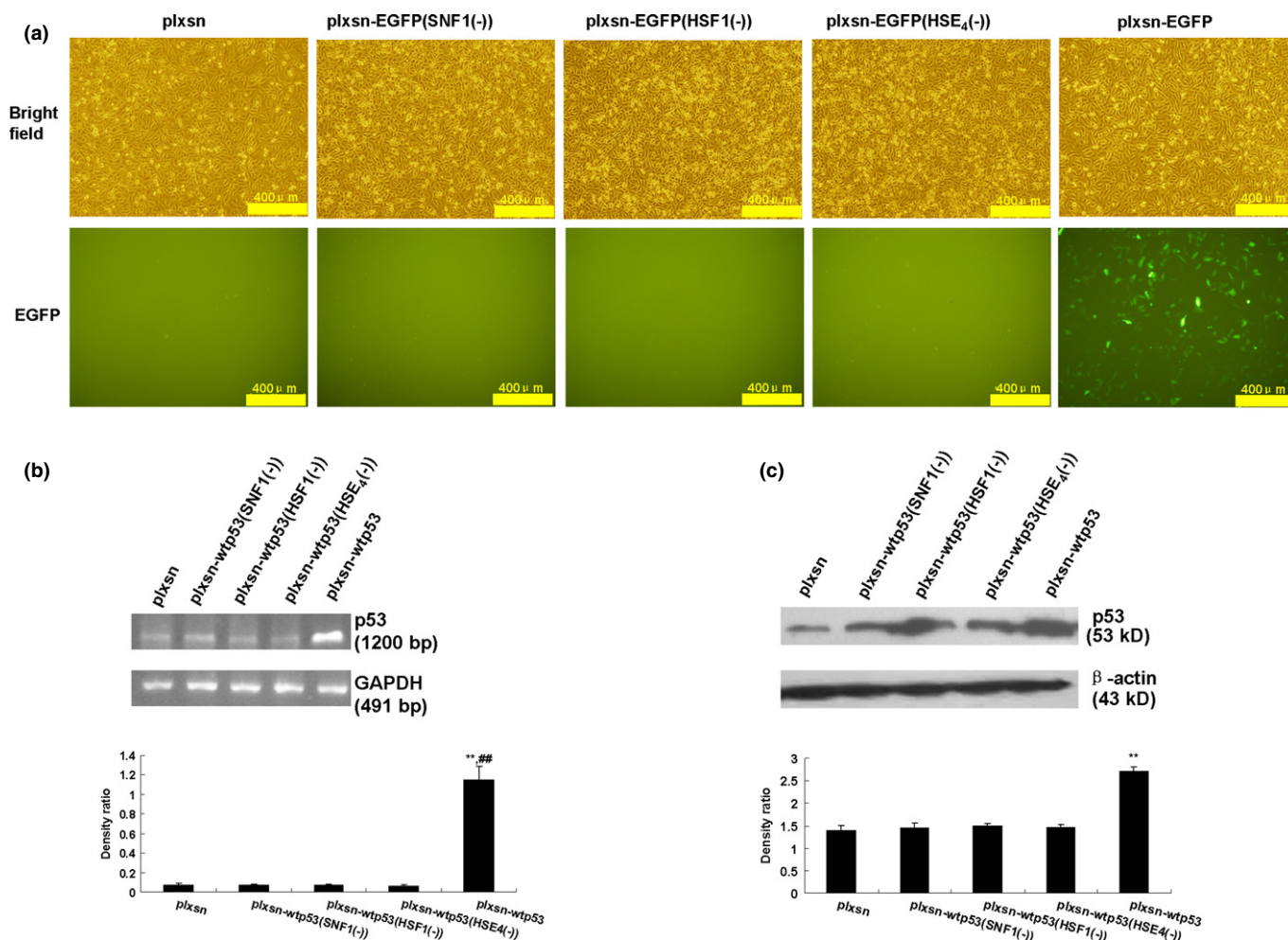
**Fig. 3.** Control experiments of engineering components identification in molecular AND gate. A549 cells were transiently transfected with plxsn, plxsn-EGFP (lacking SNF1 element), plxsn-wtp53 (lacking SNF1 element), plxsn-EGFP (lacking HSF1 element), plxsn-wtp53 (lacking HSF1 element), plxsn-EGFP (lacking $HSE_4$ element), plxsn-wtp53 (lacking $HSE_4$ element), plxsn-EGFP and plxsn-wtp53 vectors and then received 6 Gy of radiation and 1% $O_2$ treatments. After 48 h, downsteam gene was detected. (a) EGFP expression was observed with fluorescence microscope. Cells in bright fields were as control. (b) wtp53 mRNA was detected by RT-PCR and quantified ($n = 3$ for each group, with the experiments carried out in triplicate). GAPDH was as control. Data were shown as the means ± SD from three independent experiments. ** plxsn-wtp53 group compared to control groups: $P < 0.01$. (c) wtp53 protein was detected by western blotting and quantified ($n = 3$ for each group, with the experiments carried out in triplicate). β-actin was a control. Data were shown as the means ± SD from three independent experiments. ** plxsn-wtp53 group compared to control groups: $P < 0.01$.

and 3.5-fold increase on Day 13) and tumors with plxsn-wtp53 vectors injection (1.5-fold increase on Day 5 and 2.5-fold increase on Day 13). Differences of growth speed in the three groups implied that the plasmids injection might cause minor growth inhibition. Transitory inhibition of tumor growth was observed in xenografts exposed to radiation alone or injected plxsn-EGFP and exposure to radiation. During first 6 days of treatments, xenografts of the two groups grew slightly, but after treatments, the tumor grew promptly and exhibited a nearly 3-fold increase in mean tumor volume on Day 13. The data told us that radiation produced a temporal growth delay of tumors and could not affect overall tumor growth. Significant tumor regression was observed in tumors receiving plxsn-wtp53 injection and radiation. As expected, although xenografts grew slightly during the 6 days of treatments in this group, from 60 to 70 mm³; after treatments withdrawal, the mean volume kept decreasing along with time and became 59 mm³ on Day 13. The results were encouraging and indicated that AND gate vectors might have reached into the

tumor xenograft cells and were activated by endogenous hypoxia and exogenous radiation and further produced sustained antitumor effect.

To further confirm the molecular mechanism of AND gate *in vivo*, half of animals in each group were killed and xenografts were harvested on Day 10 to examine histologic changes. The H&E staining analysis showed that cells in A549 xenografts which received plxsn-wtp53 injection and radiation treatments presented significant apoptosis behaviors, such as cell shrinks, karyopyknosis, bubbles and obvious apoptotic bodies in cytoplasm compared to other groups (Fig. 6b). Prominent apoptotic response was further detected in the same xenograft (Fig. 6c), the number of apoptotic cells was much higher than that receiving other treatments ($P < 0.01$) (Fig. 6e). Moreover, IHC staining indicated that wtp53 protein exhibited higher expression level in xenografts that received plxsn-wtp53 injection and radiation treatments compared with other groups ($P < 0.01$) (Fig. 6d,f) and wtp53 could be observed to express in nuclear envelops and perinuclear space. The contrast difference of
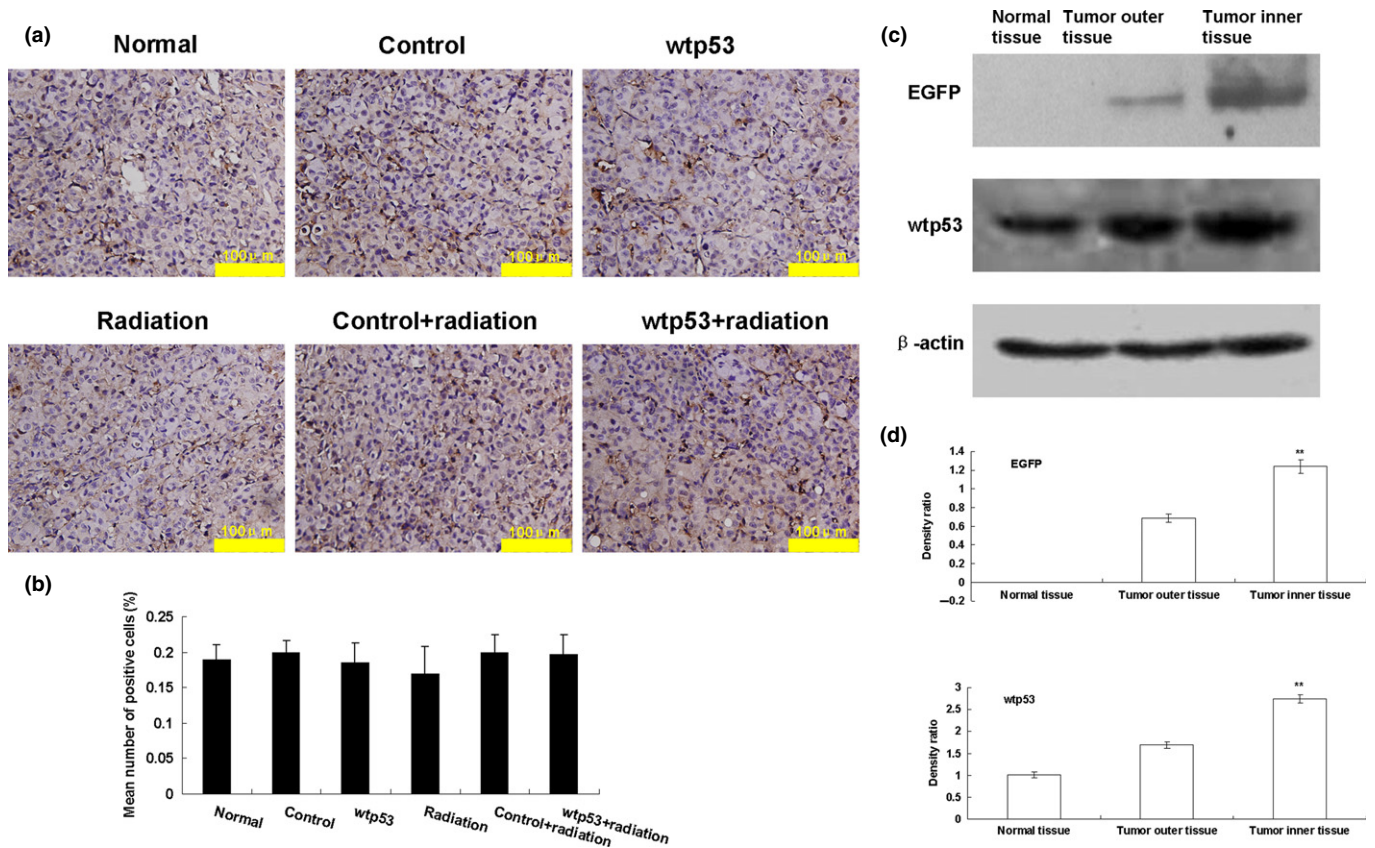
**Fig. 4.** The hypoxic areas detection and AND gate gene expression identification in nude mice xenografts. (a) Immunohistochemical detection of HIF-1α expression in A549 xenografts. (b) Mean number of positive expression of HIF-1α in different groups ($n = 3$ for each group, with the experiments carried out in triplicate). Data are shown as the means ± SD from three independent experiments. EGFP and wtp53 expression in bearing-tumors and peritumoral normal tissues received plxsn-EGFP or plxsn-wtp53 injection and focal radiation was detected by western blotting (c) and quantified (d) ($n = 3$ for each group, with the experiments carried out in triplicate). Data are shown as the means ± SD from three independent experiments. ** Central tumor group compared to control groups: $P < 0.01$. Normal: xenografts without treatment; Control: xenografts receiving plxsn-EGFP plasmids injection intratumorally on Days 1, 3, 5; wtp53: xenografts receiving plxsn-wtp53 plasmids injection intratumorally on Days 1, 3, 5; Radiation: xenografts receiving 2 Gy of radiation on Days 2, 4, 6; Control + radiation: xenografts receiving plxsn-EGFP plasmids injection intratumorally on Days 1, 3, 5 and 2 Gy of radiation on Days 2, 4, 6; wtp53 + radiation: xenografts receiving plxsn-wtp53 plasmids injection intratumorally on Days 1, 3, 5 and 2 Gy of radiation on Days 2, 4, 6.

apoptosis rate and wtp53 expression implied that although plasmids injection or radiation could induce minor cell apoptosis, activated AND gate *in vivo* could take notable antitumor effects that were consistent with the *in vitro* data.

## Discussion

The expression of therapeutic genes within peritumoral tissues to induce normal cell damage is one of the major obstacles to clinical gene therapy. Inducible promoter in gene therapy for cancer has exhibited broad potential applicability in oncology practice.[22–25] In the present study, radio-activated promoter cArG was used as transcription initiating site. Upregulation of downstream genes EGFP, SNF1, HSF1 and wtp53 demonstrated that cArG$_6$ sequences keep its radiation-induced activity when ligated to SNF1 and HSF1 genes. Moreover, apparent upregulation of EGFP and wtp53 *in vitro* and *in vivo* demonstrated the unique selective and targeting transcriptional regulation mechanism under combined stimuli.

Although inducible promoters offer the potential to selectively control therapeutic gene expression in the tumor with little normal tissue toxicity; however, when activated, these promoters often result in lower activity, and transgene expression is limited to the period of stimulation and returns to basal levels following withdrawal of the induction trigger.[26,27] In our experiments, continuous growth inhibition of xenografts with active AND gate genetic circuit demonstrated that after activation, the AND gate vectors could make persistent antitumor effects *in vivo* and might overcome the temporary expression from inducible promoter.

Aggressive tumors often have an insufficient blood supply, partly because tumor cells grow faster than the endothelial cells that make up the blood vessels, and partly because the newly formed vascular supply is disorganized.[28] Very low levels of oxygenation or hypoxia protect cells from killing by X-radiation and lead to resistance to most anticancer drugs, then accelerate malignant progression and increase metastasis.[29] Efforts to overcome the hypoxia in tumors have had only limited advancement in many studies. In current research, by using hypoxia as the optimal stimulus, we effectively transferred the adverse factor into favorable and accomplished the specific expression of ectogenic therapeutic gene. 68% apoptosis rate *in vitro* and 40% *in vivo* implied that this strategy could reach ideal therapeutic benefit.

Cancer stem cells, within the tumor population, have been demonstrated to possess extensive proliferation and
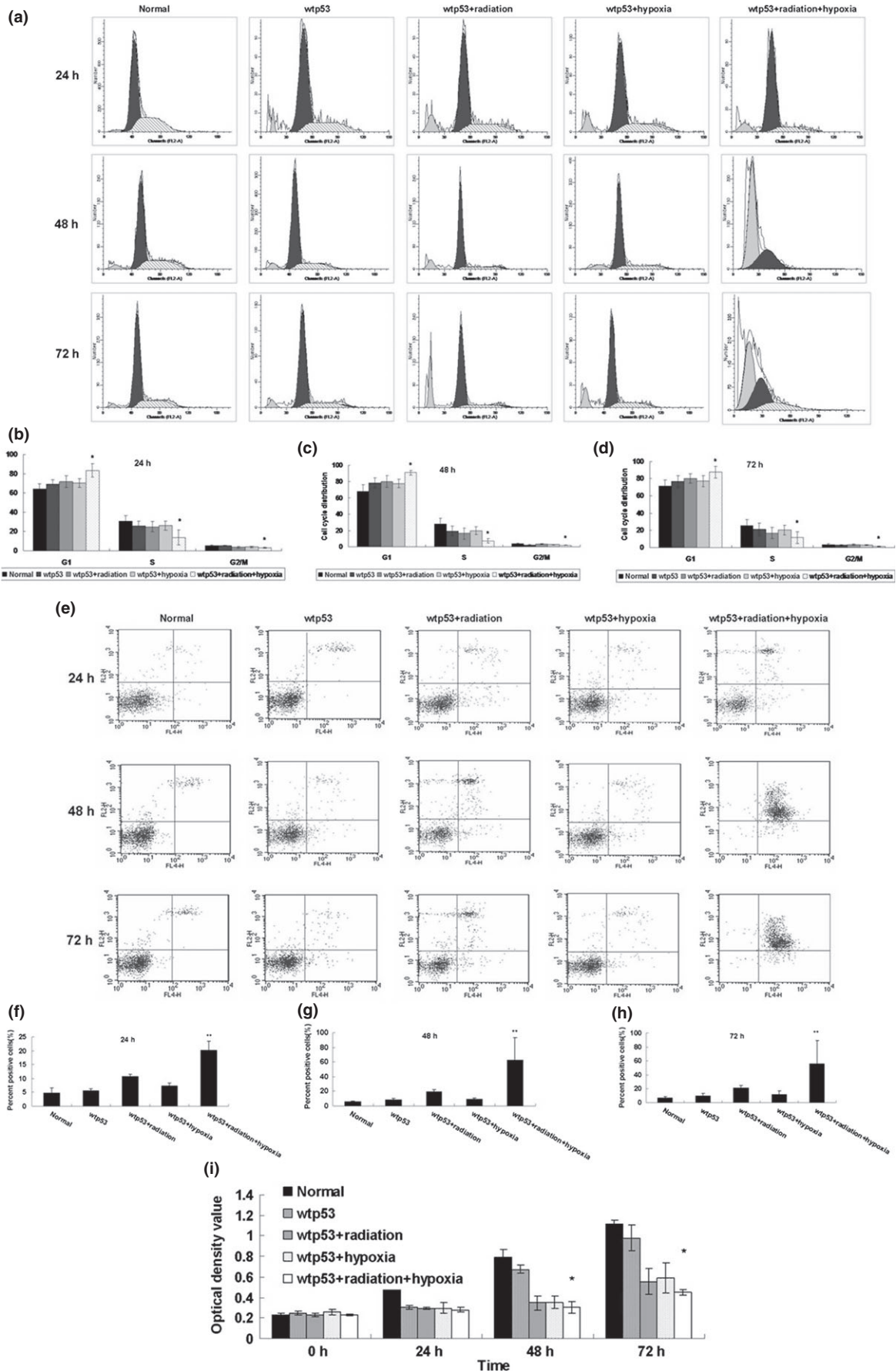
**Fig. 5.** Effects evaluation of activated AND gate through cell cycle, apoptosis and proliferation. (a–d) Cell cycle arrest was measured by flow cytometry with a FACS-Calibur (Becton Dickenson) and analyzed with ModFitLT V3.0 software ($n = 3$ for each group, with the experiments carried out in triplicate). Data were shown as the means $\pm$ SD from three independent experiments. * wtp53 + radiation + hypoxia group compared to control groups: $P < 0.05$. (e–h) FITC and PI were used to mark viable and non-viable apoptotic cells and the total apoptosis rate was detected ($n = 3$ for each group, with the experiments carried out in triplicate). Data were shown as the means $\pm$ SD from three independent experiments. ** wtp53 + radiation + hypoxia group compared to control groups: $P < 0.01$. (I) A549 cell proliferation was performed with CCK-8 assay. Relative absorbance values were shown in A549 cells receiving different treatments ($n = 25$ for each group, each group contains five samples and each sample comprises five combined cell pellets to ensure the cell numbers exceed $2 \times 10^5$ in total). Data were shown as the means $\pm$ SD from three independent experiments. * wtp53 + radiation + hypoxia group compared to control groups: $P < 0.05$. Normal: A549 cells without treatment; wtp53: A549 cells transiently transfected with plxsn-wtp53 plasmids; wtp53 + radiation: A549 cells transiently transfected with plxsn-wtp53 plasmids and receiving 6 Gy of radiation treatment; wtp53 + hypoxia: A549 cells transiently transfected with plxsn-wtp53 plasmids and receiving 1% $O_2$ treatment; wtp53 + radiation + hypoxia: A549 cells transiently transfected with plxsn-wtp53 plasmids and receiving 6 Gy of radiation and 1% $O_2$ treatments.

self-renewal capacity compared with normal malignant neoplasm and are the main cause of recurrence and metastasis of tumors. Most studies show that cancer stem cells maintain a hypoxic environment. In this research, site-specific EGFP expression induced by radiation and hypoxia could make hypoxic cancer stem cells into therapeutic areas, and through intracellular location identification, the lethal effect of therapeutic vectors targeting cancer stem cells can be accomplished.

The therapeutic gene selection is another important factor associated with the effects of gene therapy, in recent constructed models, therapeutic genes are usually prodrug-activating ('suicide') or anti-tumor genes. p53, known as a DNA damage-inducible gene, has been identified to suppress cancer progression through induction of cell-cycle arrest, apoptosis, or senescence in response to a variety of cellular stimuli.[30] However, a high percentage of human tumors are characterized by mutations of p53 that convert its tumor suppressor function into a negative action or into an oncogenic signaling coordinator with the ability to induce gene expression distinct from the wild type counterpart.[31] So, in this experiment, we chose a typical anti-tumor gene wtp53 as the suicide gene for the purpose of effective expression in tumor cells. And notable apoptosis and growth inhibition *in vitro* and *in vivo* demonstrated significantly enhanced tumoricidal activity induced by activated wtp53.

As novel gene biotechnologies, artificial genetic circuits that contain OR gate, AND gate, NOR gate and other genetic
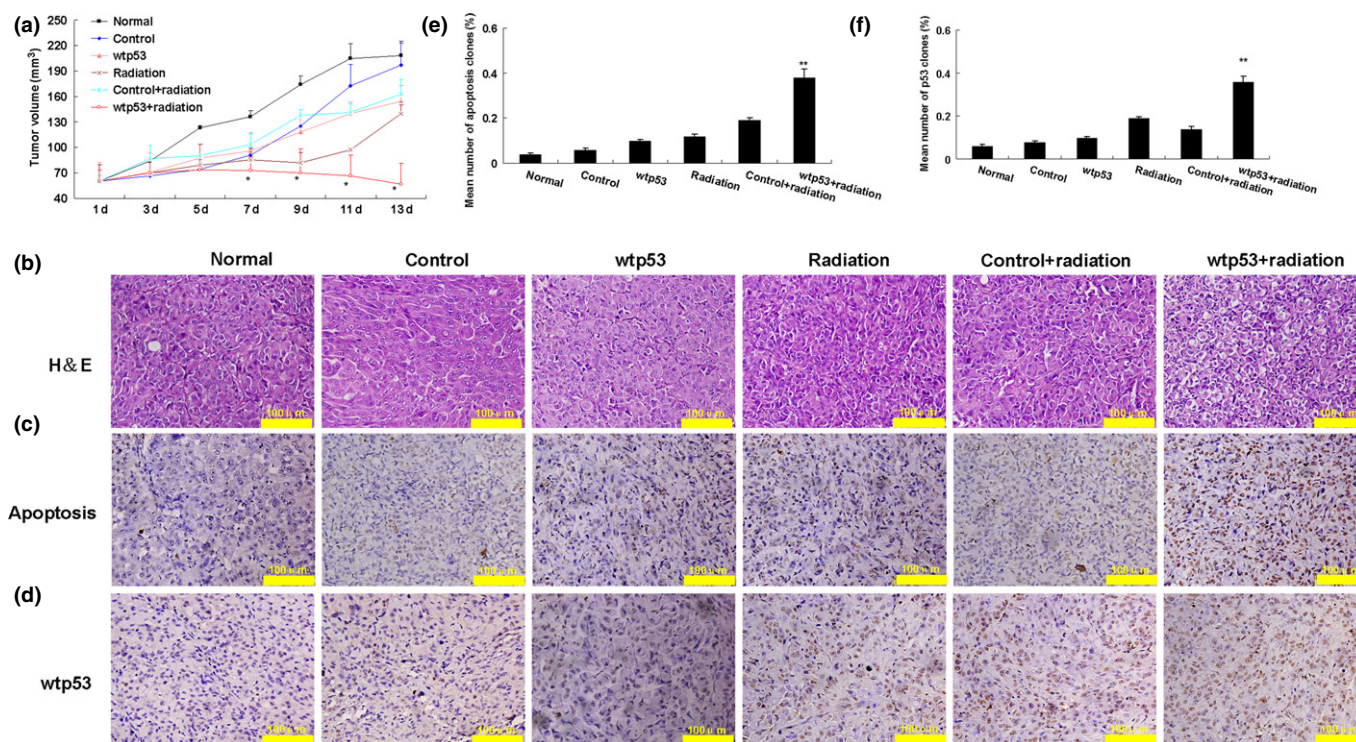


**Fig. 6.** Influence of activated AND gate to A549 xenografts. The antitumor effects of activated AND gate genetic circuit were evaluated by measuring the tumor volume and detecting apoptosis of xenografts. Day 1 represented the first day of vectors injection. (a) Tumor growth curve of different groups ($n = 5$ for each group, with the experiments carried out in triplicate). Data were shown as the means $\pm$ SD from three independent experiments. * wtp53 + radiation group compared to control groups: $P < 0.05$. (B) H&E staining of different groups. Apoptosis in situ (c) and wtp53 expression (d) in tumor xenografts were detected by immunohistochemisty. The number of apoptosis cells (e) and wtp53-positive clones (f) were quantified ($n = 3$ for each group, with the experiments carried out in triplicate). Data were shown as the means $\pm$ SD from three independent experiments. ** wtp53 + radiation group compared to control groups: $P < 0.01$. Normal: xenografts without treatment; Control: xenografts receiving plxsn-EGFP plasmids injection intratumorally on Days 1, 3, 5; wtp53: xenografts receiving plxsn-wtp53 plasmids injection intratumorally on Days 1, 3, 5; Radiation: xenografts receiving 2 Gy of radiation on Days 2, 4, 6; Control + radiation: xenografts receiving plxsn-EGFP plasmids injection intratumorally on Days 1, 3, 5 and 2 Gy of radiation on Days 2, 4, 6; wtp53 + radiation: xenografts receiving 20 µg/kg plxsn-wtp53 plasmids injection intratumorally on Days 1, 3, 5 and 2 Gy of radiation on Days 2, 4, 6.

circuits are identified to not only regulate purpose gene expression but also control expression level of the downstream gene through optimal arrangement of elements.[32–36] For example, Weiss et al.[36] constructed OR and AND genetic circuits successfully and exactly detected concentrations of toxic substances through transfection constructed vectors into Escherichia coli. But in cancer gene therapy, the use of engineering medicine has not been reported. In this research, we first constructed a specific AND gate genetic circuit which could be activated by radiation and hypoxia to accomplish optimal regulation. Our results further demonstrated that the unique transcriptional regulation mechanism of selective targeting was feasible.

In our experiments, we applied AND gate carrying wtp53 for A549 xenografts, but the vectors could also be used in other solid tumors. Furthermore, we can replace radiation and hypoxia by other stimuli such as angiogenesis or chemo-therapy agents and the heat shock response elements can also be replaced by other natural genetic circuits to construct more effective antitumor AND gates. More importantly, other anti-oncogenes or therapeutic genes can also be conjugated into AND gate vectors to produce targeting antitumor effects such as anti-HER2 gene for breast cancer.[37]

In conclusion, our radiation and hypoxia-activated AND gate might be an effective therapeutic option for patients with advanced NSCLC as well as other types of solid tumors. The low radiation dose activity also implies that this strategy may be an ideal pathway to decrease side-effects of clinical radiation therapy. The present study provides an example of overcoming hypoxia-caused radiation resistance in solid tumors. However, the vectors can not be applied for tumors with abundant oxygen and non-solid tumors such as leukemia. And the direct injection of tumors should be improved with the recent advances in radiographic imaging analysis of tumors (e.g. positron emission tomography [PET] scans) combined with computed tomography (CT) image reconstruction.

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## Disclosure Statement

The authors have no conflict of interest.

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