利用CRISPR/Cas9基因编辑系统构建 *TP53*基因敲除HeLa细胞系

董文洁[1] 杨远勤[1] 方先龙[2] 尹晓飞[2] 徐燕妮[2] 王娇娇[1] 褚 亮[2] 王如伟[3]
方 玲[3] 吴 帅[2]* 刘新垣[1,2]*

([1]浙江理工大学生命科学学院新元医学与生物技术研究所, 杭州 310018; [2]中国科学院上海生命科学研究院生物化学与细胞生物学研究所, 上海 200031; [3]浙江康恩贝制药股份有限公司, 杭州 310018)

摘要 该研究利用CRISPR/Cas9(clustered regularly interspaced short palindromic repeats/CRISPR associated protein 9)基因编辑系统构建了*TP53*(tumor antigen p53)基因敲除HeLa细胞系。CRISPR/Cas9系统能够精确地切开*TP53*基因并在双链断裂处插入选择标记(通过与供体质粒进行同源重组获得)。进一步的功能试验表明, *TP53*基因敲除的HeLa细胞拥有更强的细胞增殖能力、化疗耐药性以及氧化应激能力, 提示HeLa(*TP53*$^{-/-}$)恶性程度增强。所有的数据旨在描述一个简单和有效的方法, 即通过CRISPR/Cas9系统来构建基因缺失细胞系, 期望在较大程度上帮助研究和阐明基因功能以及细胞机制。

关键词 CRISPR/Cas9; *TP53*; 基因敲除; 同源重组修复

Knockout *TP53* of HeLa Cells by CRISPR/Cas9 Technology

Dong Wenjie[1], Yang Yuanqin[1], Fang Xianlong[2], Yin Xiaofei[2], Xu Yanni[3], Wang Jiaojiao[1], Chu Liang[2], Wang Ruwei[3], Fang Ling[3], Wu Shuai[2]*, Liu Xinyuan[1,2]*

([1]*Xinyuan Institute of Medicine and Biotechnology, Zhejiang Sci-Tech University, Hangzhou 310018, China*; [2]*State Key Laboratory of Cell Biology, Shanghai Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200031, China*; [3]*Zhejiang Conba Pharmaceutical Co., Ltd, Hangzhou 310018, China*)

Abstract We generated *TP53* knockout cells via CRISPR/Cas9 system in HeLa cells. CRISPR/Cas9 system could precisely knockout *TP53* gene in HeLa cells and insert selection markers (as donor template for the recombination repairing) in DSBs (double-strand breaks) site as well. Further functional study revealed that HeLa (*TP53*$^{-/-}$) cells get vigorous growth, resistance to chemotherapy and oxidative stress, implying that HeLa (*TP53*$^{-/-}$) cells become more cancerous. All our data described a simple and efficient approach for gene deletion in cell line via the CRISPR/Cas9 system, largely helped clarifying the functions of gene and analyzing the mechanism of cellular process.

Keywords CRISPR/Cas9; *TP53*; knockout; HDR (homology-directed repair)

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*通讯作者。Tel: 021-54921256, E-mail: hndxws@163.com; xyliu@sibcb.ac.cn
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*Corresponding authors. Tel: +86-21-54921256, E-mail: hndxws@163.com; xyliu@sibcb.ac.cn
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The pervasiveness of CRISPR/Cas9 system has brought a revolution to the genome engineering and made easier to target gene deletion. The adaptive and successive immune system, which bacteria and archaea used to direct degradation of foreign nucleic acid[1], has recently been extensively applied to facilitate site-specific DNA editing in multiple eukaryotic systems. In the type II CRISPR/Cas9 system, the Cas9 protein is subsequently recruited to the desired DNA location via the guide of crRNA, followed by a 5′-NGG-3′ protospacer-adjacent motif (PAM), then introduced DSBs (double-strand breaks) at the targeted genomic locus[2]. The DSBs would be repaired through either homology-directed repair (HDR) or nonhomologous end-joining (NHEJ) pathway in cells. In the presence of a homologous repair template, the CRISPR/Cas9 system may generate precise fragment insertion at the targeted locus through HDR, or else the DSB would be repaired through the error-prone NHEJ[3].

The transcription factor p53 has long been known as a tumor suppressor since it prevents cancer progress and formation. The p53 is essential for various cellular responses and mediates many cellular processes including growth arrest, apoptosis, differentiation, senescence and DNA damage repair[4-7]. Therefore, deficiency of *TP53* will generate phenotypic changes and even cause tumor progression[8]. HeLa cells contain wild-type p53 protein, although they contain *TP53* mRNA which is translationally active at normal level[9]. The rapid degradation of p53 by ubiquitin proteasome-mediated pathway prevents its accumulation in parental HeLa cells, so the basal p53 level was quite low[10].

Here, we generated *TP53* knockout cells via CRISPR/Cas9 system in HeLa cells, to simplify the screening process, and we provided selection markers (puromycin and EGFP) as donors for the HDR. We got the *TP53* knockout HeLa cells by screening and tested its phenotypic changes, including cell proliferation, drug resistance and oxidative stress ability. Our data confirmed the success of *TP53*-knockout cell line construction and provided a novel method to study gene function.

1 Materials and methods

1.1 Cell culture and plasmids construction

HeLa cells were cultured in DMEM with 10% fetal bovine serum (Gibco, MA, USA) and supplemented with 1% penicillin/streptomycin (Gibco), maintained at 37 °C, 5% CO_2 in a humidified incubator. The gRNAs were designed by online software (http://crispr.mit.edu/), the oligonucleotides of gRNA (sense: 5′-CAC CGT CGA CGC TAG GAT CTG ACT G-3′; antisense: 5′-AAA CCA GTC AGA TCC TAG CGT CGA C-3′) were annealed and inserted into the *Bbs* I restriction site of CRISPR/Cas9 plasmid pX330. The donor plasmid pUC19, containing puromycin resistance and EGFP, was driven by the CMV (continuous mandatory ventilation) promoter. The reporter expression cassette was PCR-amplified from pLenti-shRNA plasmid (PCR primers were listed in Table 1). Then, the constructed pUC19-EGFP-Puro plasmid was flanked each side with two homologous arms, which PCR-amplified from HeLa genomic DNA. All the recombinant plasmids were verified by sequencing.

Table 1 Primers used for plasmids construction and genotyping

Primers	Sequences (5′→3′)
GFP-puro-F	GAG ATA TCG GAG TTC CGC GTT ACA TAA CTT
GFP-puro-R	GAG ATA TCC AGG CGG GGA GGC GGC CCA AAG GG
Left-F	TGG AGC TCT GGT ACT CGC CTG TAA TCC CAG CTA CT
Left-R	TGG AGC TCT CAC ATG GCA GTG ACC CGG AAG GCA GT
Right-F	CTT CTA GAG AGG AGC CGC AGT CAG ATC CTA GCG
Right-R	CTG AAG CTT TGG TAG GTT TTC TGG GAA GGG AC
Screen-F1	TGG TGA AAC ATT GGA AGA GAG
Screen-F2	AAC CCT TGT CCT TAC CAG AAC
Screen-R	TCA ACC TCT GGA TTA CAA

1.2 Transfection and selection

Pairs of 1 μg pX330-gRNA plasmids with 2 μg donor plasmid pUC19-EGFP-Puro were co-transfected into HeLa cells by Lipofectamine 2000 reagent (Life Technologies, MA, USA) followed the manufacturer's protocols. Cells were selected with puromycin (Amresco, OH, USA) at a concentration of 2 μg/mL after transfected for 48 h, then photographed and quantified by fluorescence microscope (Nikon, Japan) and followed by FACS analysis to sort GFP-positive cells on a FACS Aria II machine (BD, Heidelberg, Germany). The sorted cells were plated in 96-well plates as monoclonal.

1.3 T7EI assay

The cells transfected with pX330-gRNA plasmid were harvested and extracted whole genomic DNA, then PCR amplified a fragment covered the cleavage site for T7 endonuclease I (T7EI) (ViewSolid Biotech, China) assay. The mutation frequency was measured via DNA bands cleavage by CRISPR/Cas-induced indels in cells.

1.4 PCR genotyping of single clones

Cells were collected and genomic DNA was extracted using the Blood Genome Extract Kit (Generay, Shanghai, China). Genomic PCR was performed with following primers which across the junction or within the deletion site (Table 1), the amplified fragment which covered the deletion region was then sequenced.

1.5 Western blot assay

Cells were harvested and lysed in RIPA buffer (Beyotime, Shanghai, China), then amounts of equal proteins were separated by 10% SDS-PAGE and transferred onto a 0.45 mm PVDF membrane (Millipore, MA, USA). Membranes were blocked for 30 min with 2% BSA (Amresco), then incubated in primary antibody for 4 h at room temperature, followed by secondary antibody for 1 h. Finally, the protein bands were detected with ECL Western blot detection reagents (CWbio, Beijing, China) according to the instructions. Antibodies of β-actin (Vazyme, Nanjing, China), p53 (Santa Cruz, TX, USA), Akt, p-Akt, p-GSK3β and p-PTEN (CST, MA, USA) were used as primary antibodies. All the secondary antibodies were purchased from Santa Cruz.

1.6 MTT assay

For cell proliferation assay, cells were seeded in 96-well plates with 3 000 cells per well and measured every 24 h by MTT assay. Briefly, added 20 μL MTT (Amresco) stock solution (4 mg/mL) to each well and incubated at 37 °C for 4 h. Then discarded the supernatants and dissolved the remains with 100 μL DMSO (Amresco) per well. After shaken for 15 min, dual wavelength of 570 nm and 630 nm were applied in absorbance assessment via a Microplate Reader (Thermo scientific, MA, USA). For drug-resistance assay, cells were plated in 96-well plates with 3 000 cells per well, then treated with 5-FU (100 μg/mL), cisplatin (10 μg/mL), doxorubincin (2 μg/mL) and rapamycin (10 μg/mL) (MCE, NJ, USA) for 48 h, respectively. For oxidative stress ability assay, cells were plated and treated as the same way with hydrogen peroxide (H_2O_2) at 0.125, 0.25, 0.5 mmol/L, then measured by MTT assay after treated for 24 h.

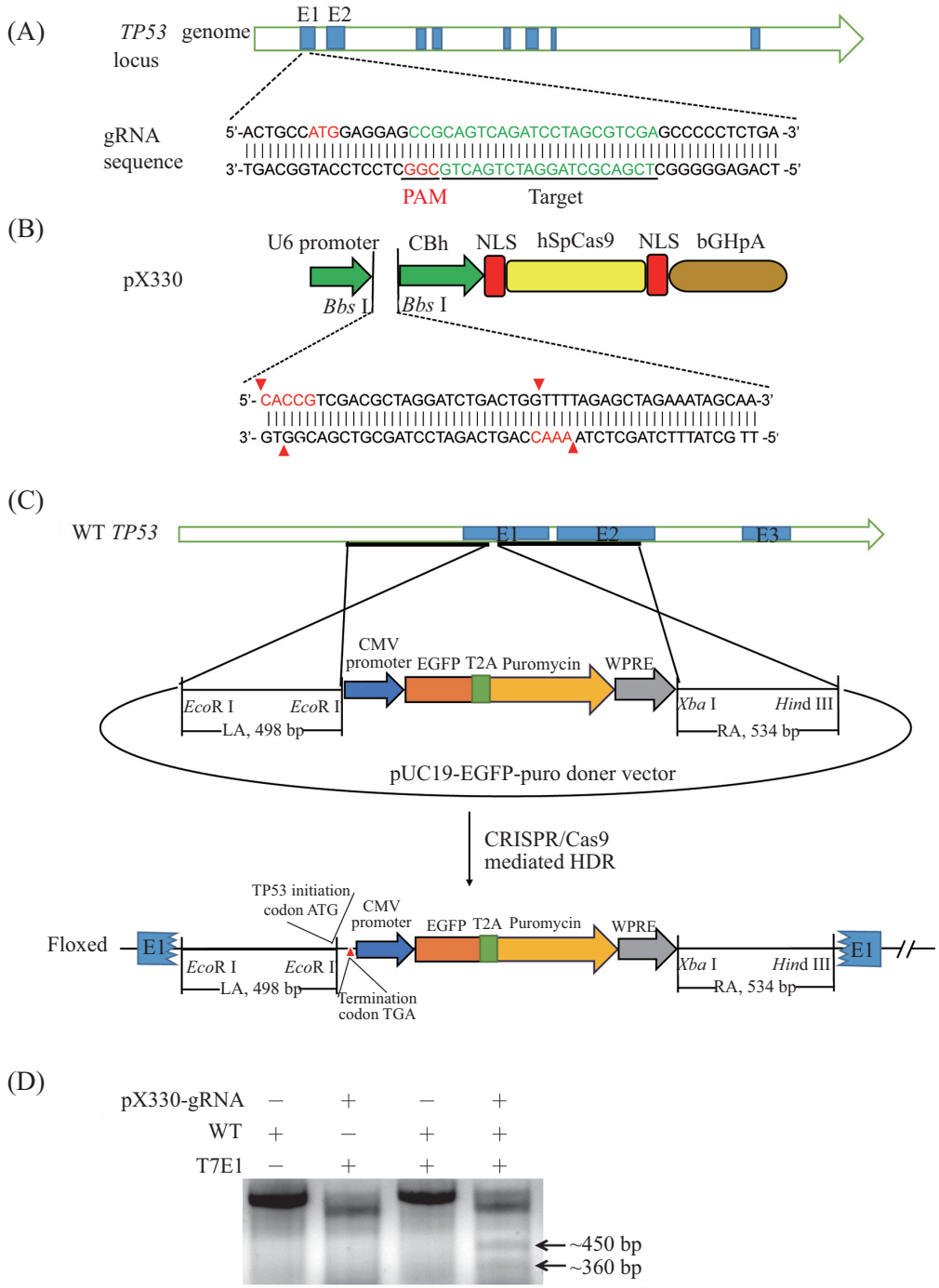
1.7 Statistical analysis

All data are shown as mean±S.E.M.. Data was analyzed by two-tails unpaired *t*-test using GraphPad Prism 5 software. $P<0.05$ was considered as statistically significant, and each experiment was repeated at least 3 times in triplicate.

2 Results

2.1 Generation of *TP53* knockout in HeLa cells

We adapted the CRISPR/Cas9 system to knockout genomic DNA in human cells. To direct Cas9 to the desired locus in *TP53*, we designed gRNAs to specifically target the downstream sequence neighboring the start codon (ATG) on exon 1 (Fig.1A). The synthetic gRNAs were annealed and cloned into pX330 (Fig.1B). To simplify the screening, a homologous recombination donor plasmid bearing the puromycin and EGFP genes was created, named pUC19-EGFP-Puro. The linearized pUC19-EGFP-Puro vector was flanked each side with 500 bp homology

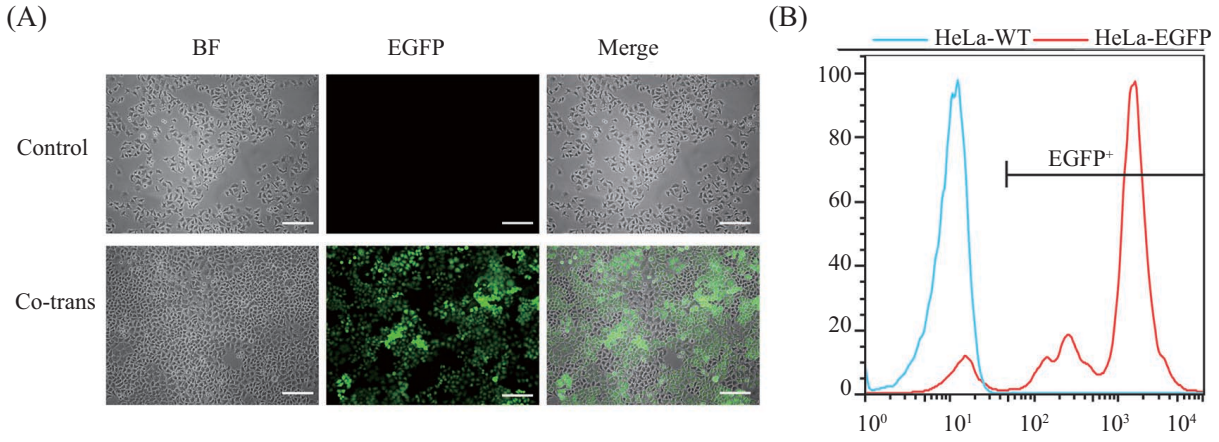


A: gRNA sequence and localization shown in relation to the *TP53* genome locus. The start codon ATG (red) is shown on the top strand. The PAM sequence (red) is shown on the bottom strand for simplicity. The target sequence (green) is shown underline. B: schematic representation of pX330 plasmid containing *TP53* gRNA. The gRNA oligonucleotides (between the 2 red triangles) are designed based on the target site sequence (~20 bp), then annealed and cloned into the pX330 vector. Sequences in red indicate the overhangs of *Bbs* I sticky ends. C: schematic diagram of the pUC19-EGFP-Puro donor vector containing 2 homology arms of ~500 bp each flanking the reporter expression cassette. A termination codon TGA is inserted on leading end of the insertion fragment (indicated as red triangles) to ensure terminating *TP53* expression strictly. LA: left homology arm; RA: right homology arm. D: detection of mutation frequency at the target site by T7E1 assay.

Fig.1 Schema for CRISPR/Cas9-mediated *TP53* knockout

arms that precisely cover the DSB region (Fig.1C).

To investigate the precise and effective genomic cleavage mediated by CRISPR/Cas9 system, we performed T7EI assay to detect the cleavage of DNA bands after transfection with pX330 plasmid in HeLa cells. According to the band intensities, mutation



HeLa cells were transfected with pX330 plasmid alone (control) or together with donor plasmid (co-trans). EGFP-positive cells were shown after puromycin selection. A: total HeLa cells at 70% confluence in bright field (BF); scale bars=200 μm. B: EGFP-positive cells were enriched and sorted by FACS.

Fig.2 Screening procedure of the desired cells

frequency was measured as 15% (Fig.1D), implied the feasibility of CRISPR/Cas9 system to knockout genomic DNA in human cells was confirmed.

To generate *TP53* knockout cells, HeLa cells were co-transfected with pX330 and pUC19-EGFP-Puro donor plasmid. Transfected cells were selected by puromycin treatment until complete cell death in control (Fig.2A). The selected cells were EGFP-positive as observed with fluorescence microscopy, which meant CRISPR/Cas9 system mediated HDR in cells successfully. The EGFP-positive cells were further sorted by flow cytometry and plated into single clones (Fig.2B).

We randomly collected 12 single clones for genotyping analysis. Genomic DNA were extracted from 12 clones and PCR-amplified fragment covering the expected cleavage sites. We used 3 pairs of primers spanning both the homologous arms and the segmental replaced regions (Fig.3A). If the wild-type allele is exist, a smaller band with a length of 433 bp would be detected (generated by the primers of screen-F1 and screen-R). Once homologous recombination occurs, an 815 bp band containing partial insertion fragment would be expanded (generated by the primers of screen-F2 and screen-R). Strikingly, PCR analysis showed that 11 clones were representing haplotype deletion genotype (+/−) for *TP53* (Fig.3B). The DSBs was largely repaired by error-prone NHEJ pathway, which frequently leading to loss (or gain) of nucleotides and resulting in gene inactivation, thus the diploid inactivation genotype (−/−) could be pick out by sequencing and Western blot analysis. Sequencing results showed that the shorter PCR products conveyed several base mutation or skipping (Fig.3C), which finally resulted in gene inactvation. To overcome low basal level, we treated the cells with cisplatin (10 μg/mL) for 24 h for increasing the p53 content, so that the amount of p53 could be detected. As evaluated by Western blot, clones 3, 5-9, 12 were still expressed the p53 protein (Fig.3D), suggesting that one of alleles was intactor merely synonymous mutation lead to incomplete deletion. The p53 protein level was undetectable in clones 1, 2, 4, 10 and 11, which indicated that we successfully constructed diploid inactivation genotype (−/−) of TP53 in HeLa cells. Indeed, the sequencing result of those clones had shown complete skipping in TP53 gene due to nucleotide insertions or deletions.

2.2 Phenotypic characterization of HeLa (*TP53*$^{-/-}$) cells obtained after CRISPR treatment

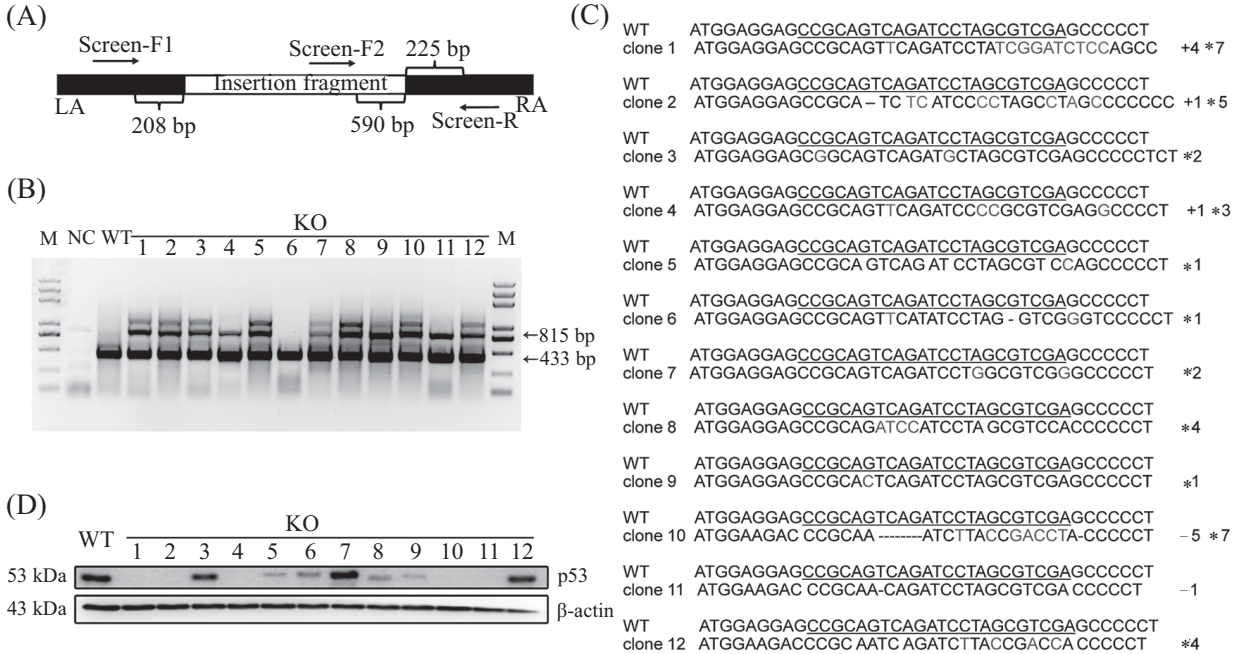
To explore the phenotypic characteristics of KO (knockout) cells, we measured the cell proliferation first. It was found that KO cells developed more vigorous growth than wild-type HeLa cells (Fig.4A). PI3K/Akt signaling pathway is the most classic signaling pathway that regulate cell proliferation[11-12]. We preferred to test whether *TP53* knockout promote

cell proliferation by activating PI3K/Akt signaling pathway. The results indicated that the protein levels of p-Akt and p-GSK3β were significantly up-regulated and p-PTEN was down-regulated in KO cells (Fig.4B).

Previous works had demonstrated that cells with mutated *TP53* or *TP53*-null were comparatively resistant to H_2O_2-induced or cisplatin-induced oxidative damage[13-14]. So *TP53* mutation is essential for oxidative damage resistance in various cancer cells. Indeed, KO cells showed higher resistance to H_2O_2-induced cell oxidative damage when treated cells with H_2O_2 at incremental concentration (Fig.4C). *TP53* mutation is also one of the reasons for cancer cell to acquire drug-resistant ability[15-16]. In our study, cells were treated with 5-FU, cisplatin, doxorubicin or rapamycin for 48 h, the KO cells presented significant drug-resistance when compared with the wild-type HeLa cells (Fig.4D). Since hypoxic inducible factor 1α (HIF-1α) has long been involved in preventing cells from apoptosis when exposed to oxidation[17], we then analysis the expression of HIF-1α. The result indicated that expression of HIF-1α was indeed increased in KO cells (Fig.4E). Of note, as the classic downstream molecules of *TP53*, mRNA levels of *Bax* and *Bcl-2* were then detected. The *Bcl-2*/*Bax* ratio showed a significant increase in KO cells than wild-type HeLa cells after 5-FU treatment (Fig.4F), which suggested that knockout *TP53* leading to multi-drug resistance may related to mitochondria apoptosis pathway.

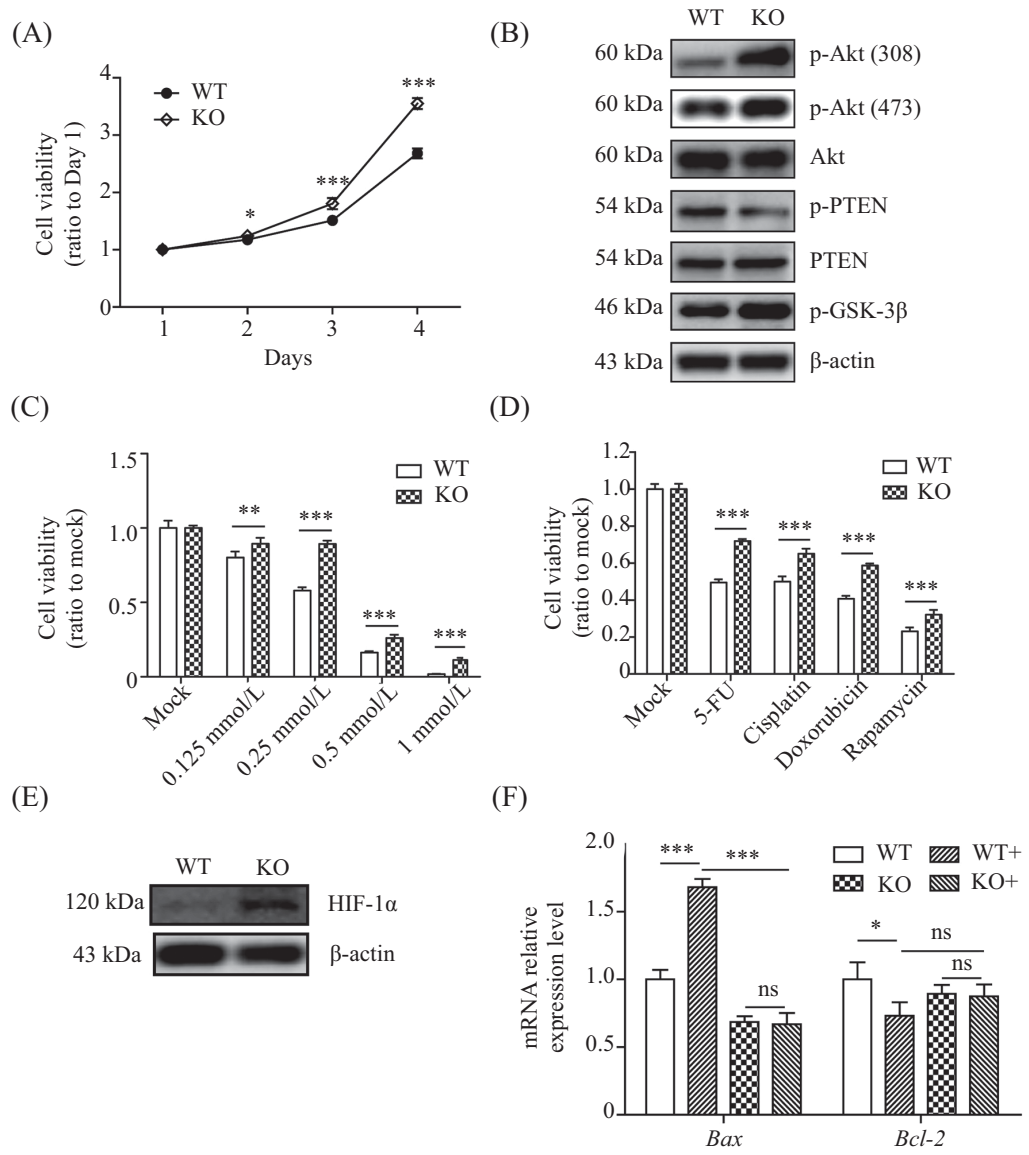
3 Discussion

Genetic deletion is indispensable in clarifying the functions of gene and analyzing the mechanism of cellular process. We successfully developed a more efficient method to knockout *TP53* in HeLa cells. Our result confirmed that the CRISPR/Cas9 system is a robust tool for gene deletion on human cells. It is concluded from the previous study that *TP53* deletion has a cardinal effect on the clinical outcome (chemotherapy resistance, overall median survival). Depletion of *TP53* that frequently occur in a number of different human cancers thus cause the loss of tumor



A: schematic diagram for the PCR detection of the deletion of the 12 selected clones. The black arrows indicate the position of PCR primers. The braces indicate insertion fragment and homologous arms amplification. Primers F1/R were used to amplify non-HDR (433 bp) and F2/R to insertion fragment (815 bp). B: PCR amplification for the 12 selected clones. The 2 black arrows indicate the 2 different amplicons mentioned in (A). M: marker; NC: negative control; WT: wild type; KO: knockout. C: DNA sequencing result analysis on the non-inserted cleavage site. +: base insertion; –: base deletion; *: base mutation. D: Western blot detection of p53 in the 12 selected clones.

Fig.3 Identification of HeLa (*TP53*$^{-/-}$) cells by genotyping and Western blot



A: cell viabilities of HeLa (*TP53*$^{-/-}$) and HeLa cells were examined by MTT assay. The experiment was repeated 3 times and values are shown as fold changes versus Day 1 group. B: Western blot detection of phosphorylated Akt, PTEN and GSK-3β in HeLa (*TP53*$^{-/-}$) cells. C: chemoresistance of HeLa (*TP53*$^{-/-}$) cells were treated with different cytotoxic chemotherapy drugs for 48 h was measured by MTT assay. D: resistance ability to oxidation stress of HeLa (*TP53*$^{-/-}$) cells were treated with H_2O_2 at various concentration for 24 h was measured by MTT assay. E: Western blot detection of HIF-1α in HeLa (*TP53*$^{-/-}$) cells. F: quantitative RT-PCR detection of *Bcl-2* and *Bax* in HeLa (*TP53*$^{-/-}$) cells treated with 5-FU for 24 h. Values were normalized to GAPDH and relative to wild-type group without treatment. +: 5-FU treatment. Representative data of 3 independent experiments was shown. **P*<0.05, ***P*<0.01, ****P*<0.001 compared with WT group.

Fig.4 Phenotypic characterization of HeLa (*TP53*$^{-/-}$) cells

suppressor activity[18-19]. In detail, *TP53* knockout often represented as inducing changes in cell cycle arrest, apoptosis, senescence, DNA repair or metabolism, which is consistent with our result.

The repair of DSBs that cut by Cas9 is largely mediated by error-prone NHEJ pathway. However, the HDR ratio is significantly increased when the homologous recombination template is exists. We optimized the screening by insert reporter tags into homologous recombination template. Usually, only one allele is repaired by HDR and another is repaired by NHEJ pathway when DSB is created by Cas9. Indeed, all the clones we tested were heterozygous insertion, which could be caused by the low transfection efficiency, or the limited recombination templates that result in finite HDR ratio. In addition, the exogenous

donor may recognized by gRNA and cut by Cas9 protein, thus the broken donor would no longer generate HDR. Modifications can be made to improve the transfection efficiency, and stabilization of donor need to be further increased.

Functional analysis revealed that the KO cells conclusively obtain several phenotypic changes, including elevated cell proliferation, resistance to chemotherapeutic drugs and oxidation. PI3K-Akt pathway is an established pathway that promotes cell proliferation[17]. In our study, we observed that the PI3K-Akt signaling pathway was activated in KO cells, which partially explained the more vigorous growth in *TP53* knockout HeLa cells. Bcl-2 and Bax were defined as anti-apoptotic and proapoptotic proteins, respectively[20]. *TP53* modulate apoptosis mainly by change the Bcl-2/Bax ratio through mitochondria apoptosis pathway[20-21]. We found that the ratio of Bcl-2/Bax was increased after knockout of *TP53*, which provides an explanation to chemotherapeutic drug resistance[22]. In addition, the KO cells got higher resistance to H_2O_2-induced cell oxidative damage, which was consistent with the Datta's work[14].

To gain new insights into the drug-resistance mechanisms in cancer cells without TP53, further microarrays analysis need to perform between TP53 KO and wild type HeLa cells when treated drugs. In addition, we could use this KO model to widely search for factors or associated signaling pathways that inhibit both invasive and metastatic processes in *TP53*-null cancer cells, which will be benefited for finding new therapeutic targets.

In general, our data further confirmed that the CRISPR/Cas9 system was an excellent technique for *TP53* knockout in HeLa cells. This method could be potentially used to target any genomic loci and should be highly valuable for studying gene function. Since *TP53* was a tumor suppressor gene, knockout *TP53* in HeLa cells resulted in growth vigorous, chemotheraputic drug resistance and oxidative stress resistance. All these data demonstrated the *TP53* knockout HeLa cells became more cancerous, which could also used as *TP53*-null model for screening of potential target.

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