ORIGINAL ARTICLE

Development of a one-plasmid system to replace the endogenous protein with point mutation for post-translational modification studies

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Abstract

Background Post-translational modification (PTM) is one of the major regulatory mechanism for protein activities. To understand the function of PTMs, mutants that prevent or mimic the modification are frequently utilized. The endogenous proteins are usually depleted while the point mutations are expressed. A common strategy to accomplish these tasks includes two-steps: First, a cell line stably expressing shRNA for protein depletion is generated, then an RNAi-resistance construct is introduced to express mutant. However, these steps are time- and labor-consuming. More importantly, shRNA and mutant protein are frequently expressed in different cells at different time, which significantly disturbs the conclusions.

Methods To overcome these technical problems, we developed a lentiviral based one-plasmid system that allowed concurrent expression of shRNA and mutant protein. The puromycin-resistant gene was inserted for the selection of stable-expression cells.

Results Using this plasmid, we efficiently replaced the endogenous proteins with comparable levels of exogenous proteins for LDHB and PKM2, two glycolytic enzymes regulated by PTM in cancer cells. The system was also successfully exploited in evaluating the role of phosphorylation of LDHB serine 162 in multiple in vitro and in vivo assays.

Conclusion Thus, we have developed an efficient one-plasmid system to replace endogenous protein with point mutations for the functional study of PTM.

Keywords PTM · Point mutation · Knockdown · pGIPZ vector · Stable expression

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Introduction

Post-translational modifications (PTMs) such as phosphorylation, acetylation and glycosylation are covalent and enzymatic modifications of polypeptide chains. PTM is one of the major regulatory mechanisms for protein functions. In response to environmental and internal signals, PTMs alter the conformation and/or the activity of the proteins, which modulate their functions in a number of biological processes, including cell cycle progression, immune responses, DNA damage repair and carcinogenesis [1]. Aberrations in the patterns of PTMs are common characteristics of cancer cells [2, 3], which are linked to the prognosis of various types of tumors [4–6]. Therefore, investigation the function of PTMs is crucial to understand the regulation of a protein. One of the most daunting challenges in PTM studies is to decipher their function in native contexts in cell culture and model organisms.

The functions of PTMs in the protein stability, activity, localization and inter-molecular interactions are investigated through expression of mutants that mimic or disrupt the modification effects. The endogenous proteins are usually depleted while the mutants are expressed. There are several methods to express the mutant and simultaneously deplete the endogenous protein in cells. Dual inducible systems [7], three-plasmid system [8], and CRISPR/Cas9 technology [9] are common methods to introduce the mutants into cells. Two-plasmid system is usually used to generate a stable cell line expressing shRNA to knockdown the target protein, then a construct containing shRNA-resistance cDNA is introduced to express the mutant. The subsequent screening of stable-expression clones would take up to several weeks, making the whole process time- and labor-consuming. Notably, the expression of shRNA and mutant is frequently non-simultaneous in individual cells, which compromised the phenotypes and statistics of the experiments. Thus, these technical problems show the need of developing a new strategy.

To overcome these technical difficulties, we introduced a one-plasmid system that facilitates the depletion of the target genes and simultaneous expression of mutants. Additionally, the levels of exogenous proteins were comparable to the endogenous ones. Cells that stably expressing shRNA and the mutant could be selected with puromycin. This system has been successfully exploited in several in vitro and in vivo assays, including enzymatic measurement, proliferation curves and xenograft models to explore the role of phosphorylation of LDHB serine 162 [10]. Thus, this one-plasmid system could be used to replace the endogenous protein with the mutant in the functional study of PTMs.

Material and methods

Cell culture

Hela, HCT-116, DLD1, U251, H1299, Hep-G2 (obtained from the American Type Culture Collection (ATCC), Manassas, VA, USA) and HEK-293T cell lines (obtained from Cell Bank, Shanghai Institute for Biological Sciences) were cultured in a 5% CO_2 humidified incubator at 37 °C and were maintained in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY, USA) supplemented with 5% fetal bovine serum and 5% newborn calf serum (Gibco, Grand Island, NY, USA), 100 units/cm^3 penicillin and 100 g/cm^3 streptomycin (Beyotime Biotechnology, Jiangsu, China).

Sub-cloning of Flag-rLDHB insert into pGIPZ vector by replacing tGFP

Flag-rLDHB cDNA was cloned from Flag-rLDHB plasmid (containing the LDHB shRNA resistance sequence). *Xba*I restriction enzyme site at the 5′ end and BamH1 restriction enzyme site at the 3′ end were introduced through PCR. The CMV promoter fragment was cloned from the pGIPZ vector. *Xba*I restriction digestion site at the 5′ end of CMV was mutated through PCR. In addition, the 17- to 19-bp homology arm sequences were incorporated in to the primers, listed in Table 1. One-step cloning method was used to replace tGFP. pGIPZ vector was double-digested with *Xba*I and Not I to linearize, and the large fragment was recovered. Then the cloned Flag-rLDHB cDNA, CMV promoter fragment and pGIPZ recovered fragment were ligated by DNA

Table 1 Sequencing primers and shRNA oligonucleotides used in this study

Sequencing primers (5′→3′)	
CMV-F	TGCAGGTCCGAGGTTCTAGCGTATTACCGCCATG
CMV-R	GGTGGCTCTAGAAGATCCTCTAGTAGAGT
shRNA oligonucleotides (5′→3′)	
shLDHB-b	AGTGAAGCCACAGATGTATAGCCCAGTTGGTATATCCATGCCTACTGCCTCGA
shLDHB-c	CACTATAGCCCAGTTGGTATATCCCGCTCACTGTCAACAGCAATATACCTTC
shLDHB-d	CGCGTCGAGGCAGTAGGCATGGATATACCAACTGGGCTATACATCTGTGGCTT
shPKM2-a	TCGAGAAGGTATATTGCTGTTGACAGTGAGCGACCATCTACCACTTGCAATTATT
shPKM2-b	AGTGAAGCCACAGATGTAATAATTGCAAGTGGTAGATGGCTGCCTACTGCCTCGA
shPKM2-c	CACTAATAATTGCAAGTGGTAGATGGTCGCTCACTGTCAACAGCAATATACCTTC
shPKM2-d	CGCGTCGAGGCAGTAGGCAGCCATCTACCACTTGCAATTATTACATCTGTGGCTT
Primers for cloning shRNA resistant target gene (5'→3')	
rPKM2-F	GATCTTCTAGAGCCACCATGGACTACAAAG
rPKM2-R	AGGGGCGGAATTTGCGGCCGGATCCTCACGGCACAGGAAC
rLDHB-F	GATCTTCTAGAGCCACCATGGACTACAAAG
rLDHB-R	AGGGGCGGAATTTGCGGCCGGATCCTCACAGGTCTTTTAG

fragment recombination using one step cloning (Vazyme, one step cloning kit).

Insertion of LDHB shRNA into pGIPZ-shLDHB plasmid

Four oligonucleotide sequences (a, b, c and d) were synthesized as listed in Table 1. Then, a/c, b/d were annealed and paired. pGIPZ-Flag-rLDHB was double-digested with *Xho*I and *Mlu*I, and the large fragment was recovered. Next, the a/c, b/d annealing products and pGIPZ-Flag-rLDHB recovered fragment were ligated by T4 ligase.

Lentivirus preparation, infection and selection of infected cells

HEK-293 T cells were cultured into a 60 cm^2 dish 24 h before transfection. One hour before transfection, the medium was replaced with fresh medium. 18 μl of PEI was added into 182 μl DMEM without serum and p/s, then mixed well by vortex and let it stand for 5 min. pGIPZ-Flag-rLDHB-shLDHB (3 μg), together with psPAX2 (1.5 μg) packaging and pMD2.G (1.5 μg) envelope plasmid DNA, was added to the mixture, mixed well and waited for 15 min. Mixture was added to HEK-293 T cells by dropwise. After 4 h of the post-transfection, the medium was replaced with DMEM supplemented with 10% FBS and p/s. After 48 h, the medium containing viruses was collected and filtered through a 0.22um PVDF filter. Required cancer cells were infected with the filtrate and polybrene (8 g/cm^3) was added for 24 h and later replaced by fresh medium. After 12 h of medium exchange, puromycin was added to kill non-infected cells.

Western Blot analysis

For western blotting, whole-cell lysates were boiled at 95 °C for 5 min and separated on 10% SDS–PAGE gels followed by transfer to PVDF membranes (Millipore, Bedford, MA, USA). The membranes were blocked with 5% non-fat milk in Tris-buffered saline containing 0.1% Tween 20 for 1 h and then incubated with primary antibody (LDHB, Proteintech 19,988–1-AP, 1:5000, LDHA, Proteintech, 19,987–1-AP, 1:5000, PKM2, Proteintech 60,268–1-Ig, 1:5000) and secondary antibodies respectively. Signals were detected by using Western Lightning Chemiluminescence Reagent Plus (Advansta, Menlo Park, CA, USA).

Measurement of LDHB enzyme activity

LDHB enzyme activity was determined by using the kit (Bioassay, D2DH-100) according to the manufacturer's instruction.

Cell proliferation and clone formation assay

For cell proliferation assay, 1000 cells were seeded in 6 well plates, and the number of cells was counted after 48 h over the 10 days. For clone formation assay, 5×10^3 cells were seeded in six-well culture dishes. After 10–14 days, cells were fixed in 4% PFA and stained with 0.2% crystal violet. Time course experiments were repeated three times.

Xenograft models

All experimental procedures involving mice were carried out as prescribed by the National Guidelines for Animal Usage in Research (China) and were approved by the Ethics Committee at the University of Science and Technology of China. The assay was conducted as described previously [10]. Briefly, 5×10^6 Hela knockdown and rescue stable cell lines were injected subcutaneously into the left or right flank of 5-week-old male BALB/c nude mice purchased from SHANGHAI SLAC LABORATORY ANIMAL COMPANY. After 4 weeks, the mice were sacrificed and photographed.

Statistical analysis

The experiments were repeated at least three times. Statistical analyses were carried out by GraphPad Prism 5 software (La Jolla, CA, USA). The data were presented as Mean $\pm$ SD. Student's t-test was used to calculate p values. Statistical significance is displayed as * $p < 0.05$, ** $p < 0.01$. *** $p < 0.001$, NS: not significant. For tumor xenograft studies, five mice were used for each group.

Results

Strategy for the construction of one-plasmid system

To overcome the disadvantages of the two-plasmid system, we sought to develop a one-plasmid system. The pGIPZ vector (Thermo Scientific Open Biosystems) was selected, because in addition to the high efficiency in transcription of shRNA, pGIPZ construct has a bicistronic transcript, which allows the concurrent expression of mRNA of tGFP and shRNA transcripts in the same cell. Based upon this advantage, we replaced the cDNA sequence of tGFP with the shRNA-resistant cDNA of PKM2 and LDHB (pyruvate kinase M2 and lactate dehydrogenase B, used as the target genes in the test), which overcame the limitations of using knockdown and rescue constructs separately. Schematic presentation of one-plasmid system used in this study is represented in (Fig. 1a-c). This new pGIPZ based one-plasmid system enabled simultaneous expression of shRNA
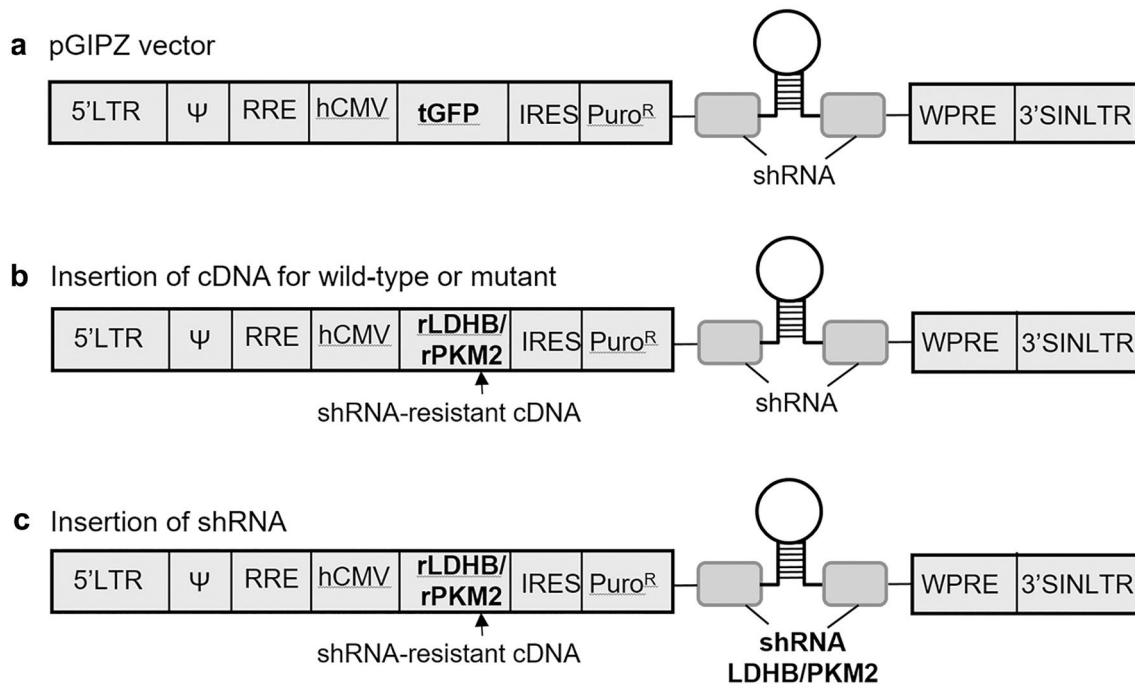
Fig. 1 Strategy for the construction of one-plasmid system. **a** The backbone of pGIPZ vector used for lentiviral expression of shRNA. **b** Replacement of tGFP coding sequence with shRNA-resistant cDNA sequence of wild-type/mutant. **c** Substitution of target gene shRNA to knockdown endogenous expression of target gene. Abbreviations of elements: **5' LTR** (5' long terminal repeat), **Ψ** (Psi packaging sequence allows viral genome packaging using lentiviral packaging systems), **RRE** (Rev response element enhances titer by increasing packaging efficiency of full-length viral genomes), **hCMV** (Human cytomegalovirus promoter), **tGFP** (Turbo GFP), **IRES** (Internal ribosomal entry site), **shRNA** (short hairpin RNA), **WPRE** (Woodchuck hepatitis posttranscriptional regulatory element enhances transgene expression in the target cells), **3' SIN LTR** (3' self-inactivating long terminal repeat for increased lentivirus safety), **PuroR** (Puromycin resistance).

and shRNA-resistant wild type or mutant, which could be verified by western blot.

To insert the mutant of target gene into pGIPZ plasmid, *Xba*I and *Bam*HI restriction enzyme sites were introduced. At 5' end of tGFP in pGIPZ, no suitable restriction site was available, therefore *Xba*I site present in CMV promoter at 5' and *Not*I at 3' end of tGFP in pGIPZ were used to linearize the plasmid by restriction enzymes. Then, an additional 17- to 19-bp homology arm sequences were incorporated into the primers. Flag-rLDHB cDNA and CMV promoter fragments were amplified by PCR. Finally, two PCR fragments and linearized pGIPZ vector were recombined using a one-step cloning assay. The new constructed plasmid was named pGIPZ-Flag-rLDHB/PKM2 (Fig. 1b).

To knock down the endogenous protein, the corresponding LDHB/PKM2 shRNA was inserted into pGIPZ-Flag-rLDHB/rPKM2 (Fig. 1c). As pGIPZ shRNAmir was based on microRNA-30 backbone, LDHB/PKM2 shRNA was inserted into the shRNAmir backbone. Four oligonucleotide sequences (a, b, c, d) were synthesized, annealed and paired. Then paired a/c and b/d were ligated into pGIPZ-Flag-rLDHB/PKM2 by *Xho*I and *Mlu*I restriction enzyme sites. Clones having LDHB shRNA were selected and validated by double digestion with *Xho*I and *Mlu*I. The generated one-plasmid construct was named as pGIPZ-shLDHB/shPKM2-Flag-rLDHB rPKM2 (Fig. 1c).

Concurrent knockdown and rescue with one-plasmid construct

LDHB and PKM2, two glycolytic enzymes, are frequently upregulated in cancer. Post translational modification of PKM2 and LDHB, such as phosphorylation, acetylation, oxidation and succinylation, regulate their expressions and activities [11–13]. It was reported that PKM2 mediated phosphorylation of histones H3T11 promotes cell proliferation, tumor progression and tumorigenesis [14]. Phosphorylation of PKM2 at Y105 is frequent in different cancers, and the expression of a non-phosphorylated mutant inhibits cell proliferation and tumorigenesis in HCC [15]. We reported that phosphorylation of LDHB at serine 162 significantly increase the metabolic activity of enzyme in cancer cells [10]. Therefore, we determined the ability of one-plasmid system to specifically ablate and rescue the expression of LDHB and PKM2 in HCT-116, DLD1, U251, H1299 and Hep-G2 cells. One-plasmid system encoding shRNA

targeting LDHB/PKM2 and LDHB/PKM2 cDNA resistant (resistant mutant) to shRNA named pGIPZ-shLDHB-Flag-rLDHB and pGIPZ-shPKM2-Flag-rPKM2 were constructed and transduced in different cell lines. After antibiotic selection of stable cell lines, we assessed the knockdown and rescued effect of LDHB and PKM2 in different generated stable cell lines.

The expression of LDHB was examined in HCT-116, DLD1 and U251 cell lines (Fig. 2a), while the expression level of PKM2 was examined in H1299 and Hep-G2 cell lines (Fig. 2b). The immunoblots demonstrated that the expression of endogenous LDHB and PKM2 and their expression levels were significantly reduced, and the levels of exogenous LDHB and PKM2 were comparable to wild-type LDHB/PKM2 in multiple cancer cells (Fig. 2). Thus, this one-plasmid lentiviral system allows to simultaneously knockdown the endogenous protein and rescue with comparable exogenous protein in different cancer cells.

Evaluating the function of LDHB S162 using one-plasmid system *in vitro* *and* in vivo

To test whether the one-plasmid system can be used to evaluate the function of PTM, we first confirmed that endogenous LDHB was depleted and the expression levels of exogenous wild type and mutant were comparable to the endogenous LDHB in HeLa cells (Fig. 3a). As LDHB catalyzes the conversion of lactate to pyruvate [16], the enzymatic activity of LDHB in whole cell lysate was determined. Indeed, shRNA-resistant LDHB rescued the enzyme activity of lactate to pyruvate (Fig. 3b). Moreover, the knockdown of LDHB

inhibited the cell proliferation and reintroduction of shRNA resistant LDHB-WT restored the cell growth (Fig. 3c).

We reported that phosphorylation of LDHB at serine 162 significantly increase the enzymatic activity in converting pyruvate to lactate, which promotes tumor growth [10]. Therefore, we introduced a point mutation at the PTM site of LDHB-S162A (non-phosphorylated mutant) and confirmed the proliferation of Hela cells expressing LDHB-S162A with colony formation assay in cultured cells and xenograft tumors. The growth of cells and progression of tumors was notably diminished after the depletion of LDHB. While the growth inhibition was rescued by reintroduction of shRNA resistant LDHB-WT expression but LDHB-S162A mutant expression was not restored (Fig. 3d), indicating that phosphorylation of LDHB at S162 is necessary for proliferation of tumor cells as well as in xenografts (Fig. 3e). Furthermore, immunoblot analysis clearly showed that the expression level of endogenous LDHB was significantly knocked down, and the exogenous shRNA resistant LDHB-WT and LDHB-S162A were comparable to the endogenous levels in vivo (Fig. 3f).

Taken together, these data from in vitro and in vivo assays showed that the one-plasmid system could be used to knockdown the endogenous protein and simultaneously re-express exogenous protein mutant, making it an efficient tool in the functional study of PTMs.

Discussion

One of the technical challenges in PTMs studies is to concurrently deplete endogenous protein and expression of point mutation at physiological levels. Additionally, as off-target

Fig. 2 Concurrent knockdown and rescue with one-plasmid construct. **a** HCT-116, DLD1 and U251 cell lines were transduced with pGIPZ-shLDHB-Flag-rLDHB and expression of endogenous and exogenous LDHB was examined western blot. **b** H1299 and A549 cell lines were transduced with pGIPZ-shPKM2-Flag-rPKM2 before the expressions of endogenous and exogenous PKM2 were examined by western blot. NT (non-targeting).
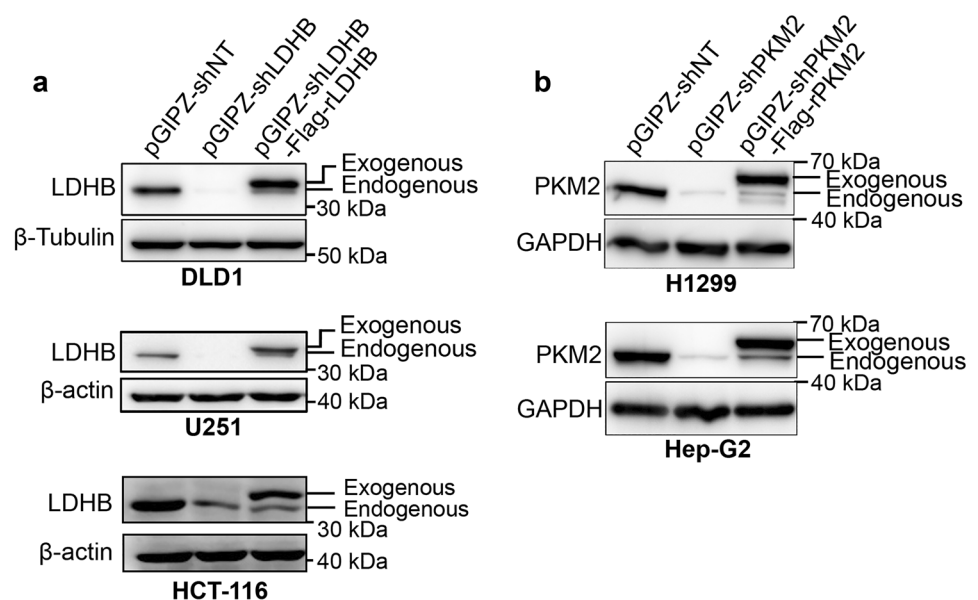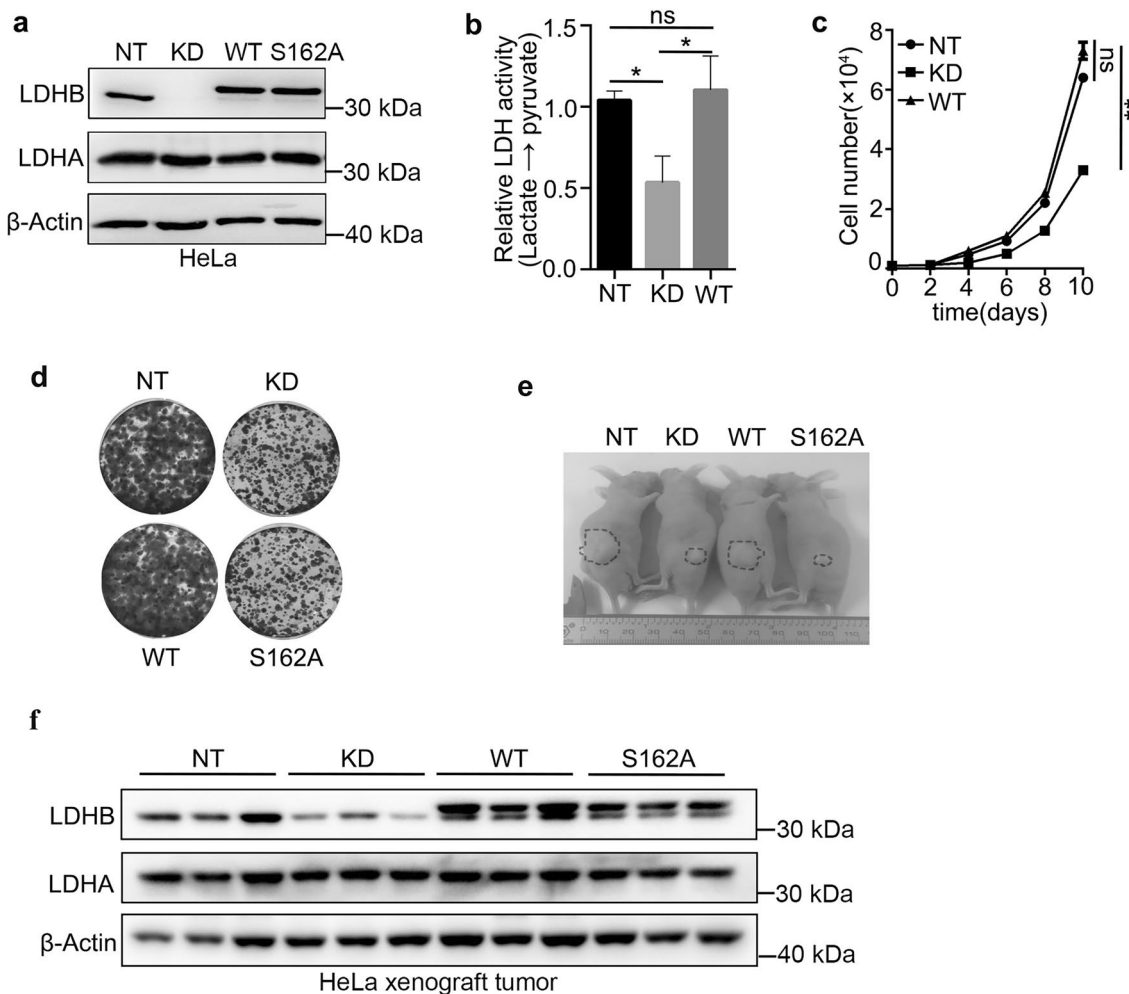
Fig. 3 Evaluating the function of LDHB S162 using one-plasmid system in vitro and in vivo. **a** Knockdown and rescue expression levels of LDHB in Hela cells examined by western blot. **b** Enzymatic activity of lactate to pyruvate was determined in whole cell lysate of Hela cells. **c** Cell proliferation assay conducted in stably expressing one plasmid system in Hela cells. **d** Colony formation assay to access the ability of LDHB S162A for formation of colonies. **e** Photograph of xenografts tumors at the endpoint. **f** Expression levels of LDHA/LDHB in xenograft tumors determined by western blot. KD (knock down), WT (wild type).

effect of RNAi is very common, it is important to perform the rescue assay by re-introducing RNAi-resistant construct to verify the specificity of the subsequent phenotype. The most frequent strategy is using separate constructs and multiple steps for knockdown and the expression [17, 18]. However, these experiments cause variances in transfection, transduction and selection efficiency for individual cells, which lead to inconsistent results. The one-plasmid system described in this study can overcome these problems with the ability to simultaneously drive the expression of a shRNA and a rescue protein expression of the target genes in the same cells.

Another advantage of this lentiviral-based one-plasmid system is the simple procedure in construct generation that includes insertion of shRNA and a one-step-cloning method for the insertion of the protein coding sequence as detailed in

Fig. 1. The duration of the experiment is much shorter than that using the two plasmid system because it only required one transduction of lentiviral particles. It takes only about ten days from the plasmid construction to the generation of the cell lines with stable knockdown and expression of rescue protein, whereas it takes at least 3–4 weeks with two-plasmid system (supplementary information). More importantly, when cDNA and shRNA are transcribed simultaneously in the same cell, the actual functions of PTMs could be evaluated using single cell or bulk cell assays.

Notably, the expression levels of wild-type and mutant could be optimized with promoter selection or positional effect. It is known that the expression levels of some genes correlate with the positions of their promoter [19]. This can also be applied for the one plasmid system by either positional cloning or promoter selection. The CMV promoter is a

strong promoter and can meet the need of most of the genes expression [20], however, if the expression of exogenous gene is much higher than the endogenous one, the CMV promoter could be replaced by weaker promoters. Additionally, if the *Xba*I and *Bam*HI digestion sites are not suitable, these enzyme sites could be replaced using PCR.

Conclusion

We generated a one-plasmid system that can rapidly, stably and efficiently replace the endogenous proteins with point mutation. This system could be a useful tool to study the functions of post translational modifications of protein in vitro as well as in vivo.

Declarations

Conflict of interest The authors have no conflicts of interest.

Ethical approval All experimental procedures involving mice were carried out as prescribed by the National Guidelines for Animal Usage in Research (China) and were approved by the Ethics Committee at the University of Science and Technology of China.

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