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Establishment and Validation of Human Colon Cancer HCT116 *wip*1 FLAG Knock-in Cell Line

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Abstract

A HCT116 cell line with FLAG Knock-in in the 5' terminus of its *wip*1 gene was established by genome editing via Adeno-Associated Virus (AAV)-mediated Homologous Recombination (HR) "knock-in" approach and identified by PCR, sequencing and western blot. The corresponding AAV-HR gene targeting can facilitate many protein functional analyses, including western blot, immunoprecipitation, and protein screening etc. This human somatic cell gene targeting technique provides a general solution not only for the study of proteins without ideal antibodies, but also for screening potential proteins interacting with targeted protein in human somatic cells.

Keywords: Genome editing; Gene knock-in; Human somatic cell gene targeting; Adeno-associated virus mediated homologous recombination (AAV-HR); HCT116 cell line; *wip*1 gene

Introduction

Knock-outs and knock-ins by homologous recombination are powerful genome editing techniques to definitively assess gene function. These have brought important insights into the genetic basis of many human diseases, e.g. cancer, heart disease and immune disease, etc. Human somatic cells' knock-out/-in can provide a genetic model system applicable to the study of virtually any cell-based phenomenon. Furthermore, they display conceptual advantages over analogous studies in animal or other model systems when the biochemical and physiologic pathways involved vary among cell types or organisms [1].

Knock-in introduces sequences into an endogenous gene which can include alterations as small as a single base substitution or as lager as a reporter gene, for example, FLAG, EGFP, HA or some other epitope tags. Homologous recombination-based gene targeting was firstly introduced into human cells by Porter and Itzhaki in 1993 [2], and since then tens of different genes (mostly cancer-related) have been modified by homologous recombination [1,2]. On account of technical difficulties, this approach has not been widely used in human cells. Ten years later, Hirata et al. [3], Porteus et al. [4] and Kohli et al. [5] successively demonstrated that the targeting vectors created in Adeno-Associated Virus (AAV) backbones and delivered by infection can result in reproducibly high efficiencies of gene targeting. Furthermore, Topaloglu et al. [6] described a NeoR gene cassette termed a Synthetic Exon Promoter Trap (SEPT) made the creation of promoter trap targeting vector simpler and more technical tractable. These advances have made it more feasible to modify alleles in human cells. Then Kim et al. [7] have FLAG tagged many genes with this technique in many human somatic cells. Of note, it has been successfully used to tag all allele of Saccharomyces cerevisiae, and the corresponding proteins' function in their natural status and interaction with other proteins were studied with western blot, immunoprecipitation and immunocytochemistry without the need for specialized antibodies to the protein of interest [8].

Presently, epitope-tagged expression vectors have been widely used to study gene function [9,10]. However, this kind of transgenes generally lack features present in endogenous genes such as a natural promoter, introns and 5'- and 3'- untranslated regions, all of which contribute to transcriptional and translational regulation. Ectopically expressed transgenes are generally expressed much higher than the endogenous gene. Therefore, results generated using epitope-tagged expression vectors are often criticized as being possible artifacts of overexpression and/or unnatural expression and generally require confirmation with endogenous proteins. It is desirable to perform epitope-tagging on endogenous genes (EET) with the above improved homologous recombination-mediated gene knock-in technique.

WIP1, the acronym of wild-type p53 induced phosphatase 1, is a nuclear serine/threonine phosphatase of the PP_2C family encoded by protein phosphatase magnesium-dependent 1 delta gene (*PPM1D*, or *wip*1). It has distinctive oncogenic properties, shown by inhibitory functions on several tumor suppressor pathways, including ATM, CHK2, p38MAPK and p53. Some newly emerging functions of *wip*1 make it a potent therapeutic target against cancer and aging [11,12]. Here, I would like to share with peers the detailed steps of AAV-HR mediated human somatic cell gene knock-in technique and its application to creation of FLAG tag of endogenous *wip*1 gene in human HCT116 cell line.

Materials and Methods

Cells and cell culture reagents

The human colorectal cancer cell line HCT116 and human embryonic kidney cell line 293T were grown in DMEM High Glucose (GIBCO®11965, Invitrogen) supplemented with 10% fetal bovine serum (HyClone®143471, Thermo Scientific) and penicillin/streptomycin (GIBCO®15140, Invitrogen).

PCR-based creation of homologous arms

Homologous fragments for creation of *wip*1 FLAG-tagging

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vectors were obtained by PCR from a human genomic DNA template using VENT polymerase (New England Biolabs) according to the manufacturer's protocol. Homologous arms were ~1kb in size, respectively. The left arm was composed of part 5′ upstream sequence, exon I / intron I, and the right arm was composed of a portion of intron I. The sequence of all PCR primers is available from the author upon request.

Site-directed mutagenesis for addition of FLAG epitope

Site-directed mutagenesis for the addition of FLAG tag to the left homologous arm of the *wip*1 vector was performed using the Quickchange Kit (Stratagene) with PAGE-purified oligonucleotides designed to add an in-frame FLAG tag (Integrated DNA Technologies, IDT). A total of 25% of colonies tested by PCR and sequencing for the *wip*1 vector, contained the desired 24-nt FLAG insertion (GAT/TAC/AAG/GAT/GAC/GAC/GAT/AAG encoding DYKDDDDK).

Assembly of FLAG-tagging vector

pAAV-SEPT- Acceptor vector was constructed by and kept in Todd Waldman's lab of Lombardi Comprehensive Cancer Center, Georgetown University School of Medicine. The PCR product composed of the left homologous arm was digested with Nhe I and Sac I, and that composed of the right homologous arm was digested with Nde I and Xhol I. The digested left and right arms were then simultaneously cloned into the pAAV-SEPT-Acceptor vector that had been digested with Nhe I, Sac I, Nde I and Xhol I with T4 DNA ligase (BioLabs). All endonucleases were obtained from New England Biolabs. After transformation into Oneshot® TOP10 chemically competent cells (Lot#768388, Invitrogen), 446 colonies were obtained, and a subset was tested by endonuclease digestion, about 1 of 8 (12.5%) colonies contained plasmids in which the ligation had successfully occurred. A subset of positive clones was further confirmed by DNA sequencing; in all cases, the junctions were correct.

Packaging of rAAV targeting constructs

Transient stocks of AAV viron were created by co-transfection of 293T cells with epitope-tagging vector together with pAAV-RC (Stratagene) and pHELPER (Stratagene) using FUGENE 6 (Roche, Indianapolis, IN, USA) as previously described [13]. Briefly, Inverted Terminal Repeat (ITR)-containing targeting constructs were co-transfected with the plasmids pAAV-RC and pHELPER. Approximately 5×10^6 AAV-293T cells in a Ø 10cm dish were transfected with a mixture of 2.5μg of each of the above three plasmids, using 18μl of FUGENE. The transfection was performed in serum-free conditions using DMEM medium (GiBCO®, Invitrogen). One day after transfection, media was aspirated and cell monolayers were scraped into 1mL sterile Phosphate-Buffered Saline (PBS) and subjected to four cycles of freeze/thaw (consisting of 10min freeze in a dry-ice ethanol bath and 10min thaw in a 37°C water bath, vortexing after each thaw). The lysate was then clarified by centrifugation at 12,000rpm for 10min in a benchtop microfuge to remove cell debris, and the virus-containing supernatant was aliquoted and stored at -80°C.

Gene targeting and isolation of recombination cell lines

HCT116 cells were grown in 25cm^2 flasks and infected with rAAV when ~60% confluent. Viruses were then added to each flask to infect cells. Cells were washed with Hanks buffered saline solution, detached with trypsin (Invitrogen) and then replated at limiting dilution into ten 96-well plates in medium containing geneticin (G418, Invitrogen) at a final concentration of 0.6mg/ml 1 day after infection. Drug resistant colonies were grown for 2-3 weeks. Individual G418-resistant clones were expanded to 24-well and 12-well plates in order and used for the preparation of genomic DNA using standard techniques. Clones were tested for homologous integration of the targeting vector using a primer pair specific for the target allele (one annealed outside the homology region and another annealed within *neo*). Neo-PCR products from clones with homologous integration of the targeting vector were then sequenced to determine whether the FLAG epitope had been inserted into the genome of each cell line.

Once individual clones were identified, they were infected with a Cre-expressing adenovirus as follows. Individual clones were expanded by limiting dilution and tested for the restoration of G418-sensitivity.

Cre-mediated excision of selectable marker elements

To remove the SEPT cassette from correctly targeted clones, cells were infected with an adenovirus that expresses the Cre recombinase. Cells were plated at limited dilution in nonselective medium 24h after infection. After 2 weeks, single cell colonies were plated in duplicate and 0.6mg/mL G418 was added to one set of wells. After 1 week of growth, G418-sensitive clones were expanded for further analysis.

Preparation of protein lysates and western blot

Protein lysates to be used for western blot were prepared in 1% NP40 RIPA buffer. Protein concentrations were quantified using the bicinchonicnic assay (Pierce, Rockford, IL, USA).

For FLAG affinity purification, α-FLAG M2 beads (SIGMA, St Louis, MO, USA) were washed once with RIPA, then re-suspended in RIPA fractions derived from parental or FLAG-tagged cells, and rotated at 4°C overnight. Beads were then washed three times with RIPA, resuspended in sample buffer and separated by SDS-PAGE.

Western blot was performed using standard techniques. The primary antibodies were FLAG polyclonal F7425 (SIGMA) and *Wip*1 polyclonal A300-664A (Bethyl). The secondary antibody was α-rabbit IgG HRP-linked antibody 7074 (Cell Signaling).

Results

Experimental design

A knock-in targeting construct contains both custom-designed and generic elements (Figures 1a and 2). The former are known as Homology Arms (HAs), flanking the selectable marker cassette, define the location into which the latter will be inserted (Figure 1b). Successful creation of a knock-in allele requires knock-in vector to make deletions fall within an intron to minimize disruption of gene structure.

HAs can be amplified from a human genomic DNA library in bacterial artificial chromosome clones, but it is also a good choice to amplify from genomic DNA of the cell line to be targeted (Figure 3).

Small genetic alterations can be engineered into the HAs (Figure 1b) by site-directed mutagenesis. Usually, the vector should be designed so that the knock-in elements is as close as possible to the

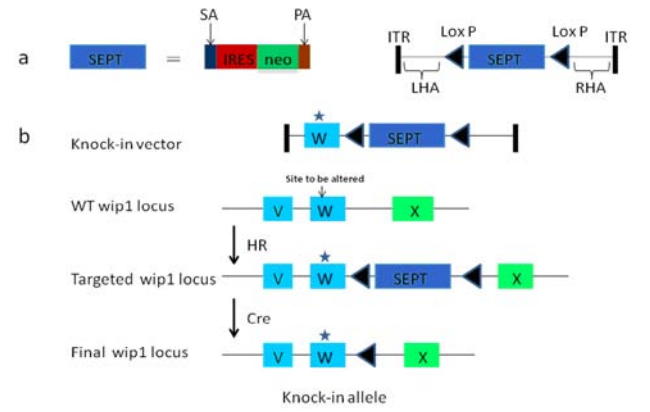

Figure 1: Overview of the generation of knock-in alleles by homologous recombination. (a) Illustration of the functional elements of a gene knock-in vector. A generic construct contains two HAs that flank a central element featuring a selectable marker gene. A versatile cassette, known as the SEPT element, contains a Splice Acceptor (SA) followed by an Internal Ribosomal Entry Sequence (IRES), the coding sequence of Neomycin transferase (*neo*) and a polyadenylation signal sequence (pA). This element is flanked by recognition sites (LoxP) for the cre recombinase in the plasmid pSEPT. Arranged in a head-to-tail configuration, the LoxP sites facilitate the removal of all marker elements. (b) Schematic illustration of gene knock-in at the *wip*1 locus. Human *wip*1 is a gene composed of 6 exons, encoding a protein of 605 Aa residues. The FLAG encoding sequence was added to the 5'-end of the coding region, or just after the starting codon ATG of exon I. The two triangles flanking the SEPT cassette represent the LoxP sites. ITR represents inverted terminal repeat.

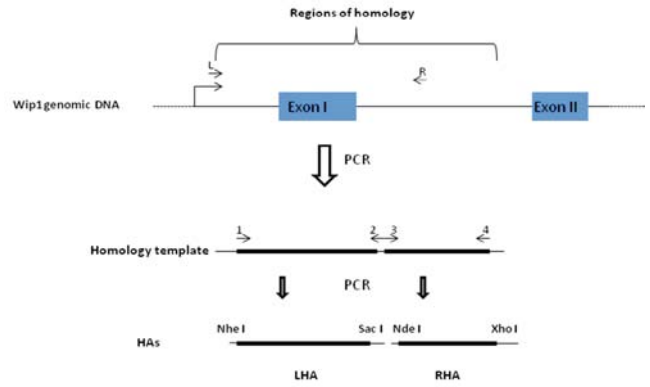

Figure 2: Synthesis of HAs. The design of HAs will determine the region of the point of insertion and is therefore of critical importance. The first stage is the generation of a homology template. Primers L and R are designed to span the entire proposed recombination region of homology. If genomic PCR generates a robust product, primers L and R can also be used as screening primers (Figure.4). Primers 1-4 are designed to amplify HAs regions within the homology template. Before vector assembly (Figure.3), embedded restriction sites are digested with restriction endonucleases Nhe I, Sac I, Nde I and Xho I.

selectable marker cassette, sometimes just outside the paired LoxP elements.

rAAV packaging capacity determines the total size of the knock-in construct. Packaging is most efficient with recombinant genomes between 4.1 and 4.9kb. HAs should be as large as possible to maximize targeting efficiency. The SEPT element, a standard selectable marker cassette is about 2kb in size. This leaves a total of 2.6kb for HAs,

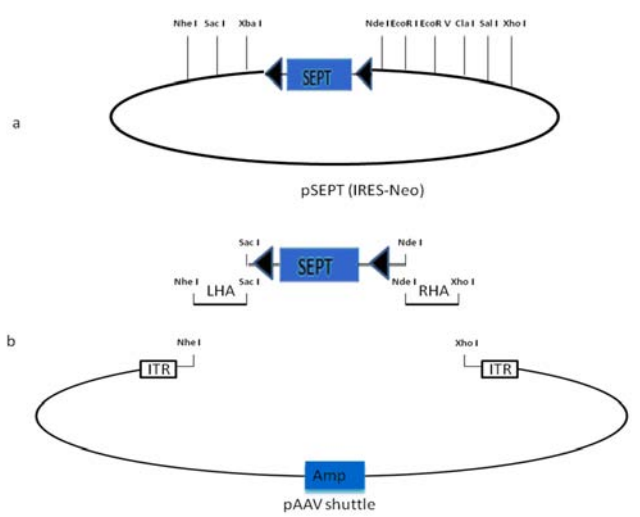

Figure 3: Assembly of a rAAV targeting construct. (a) The SEPT element and Lox P sites are flanked by several unique 6-bp restriction sites to facilitate cloning. (b) The final construct is assembled by a four-part ligation that includes the two HAs (LHA and RHA), the SEPT/LoxP cassette and a pAAV shuttle vector that contains AAV Inverted Terminal Repeats (ITRs). The SEPT element is designed to ligate to each HA through base pairing of unique restriction sites.

corresponding to 1.3kb per arm. We usually use HAs of 0.9-1.3kb. Assembly of rAAV targeting construct is shown as (Figure 2).

The generation of homologous recombinant clones with rAAV does not require the production of a purified, high-titer viral stock. When incubated with a low-titer lysate, the majority of rAAV-infected cells do not integrate viral DNA. Because the targeting construct contains a selectable marker, even rare clones with integrated transgenes can be readily expanded. Crude viral lysates have proved to be more than adequate for the generation of hundreds of transgenic clones for subsequent screening.

Recombinant clones are identified by the analysis of genomic DNA. There are several options for isolating genomic DNA from drug-resistant clones. Commercially available manifolds that employ glass milk technology such as those available from Promega and Qiagen are reliable. Crude lysates by SDS-Proteinase K, separated by high vacuum grease (Dow Corning®, Midland, MI, USA), can also provide DNA of sufficient quality for most screening purposes at a significantly lower cost.

Another critical step in the isolation of targeted cell lines involves the screen for proper integration of the construct. Several PCR-based strategies have been successfully employed to identify the cell clones that harbor recombinant alleles [1]. The simplest of these involves the amplification of a diagnostic DNA wherein one primer anneals within the selectable marker and the second primer anneals outside the homology region (Figure 4). The relatively compact HAs typically incorporated into rAAV targeting vectors to facilitate this screening approach. Of note, this approach does not allow a positive control reaction, rendering the screening process essentially "blind" until a correct recombinant is identified. A useful modification of the standard PCR-integration screen incorporates an extra 20bp at the 5' end of the primer used to amplify one of the HAs. The extra 20bp are

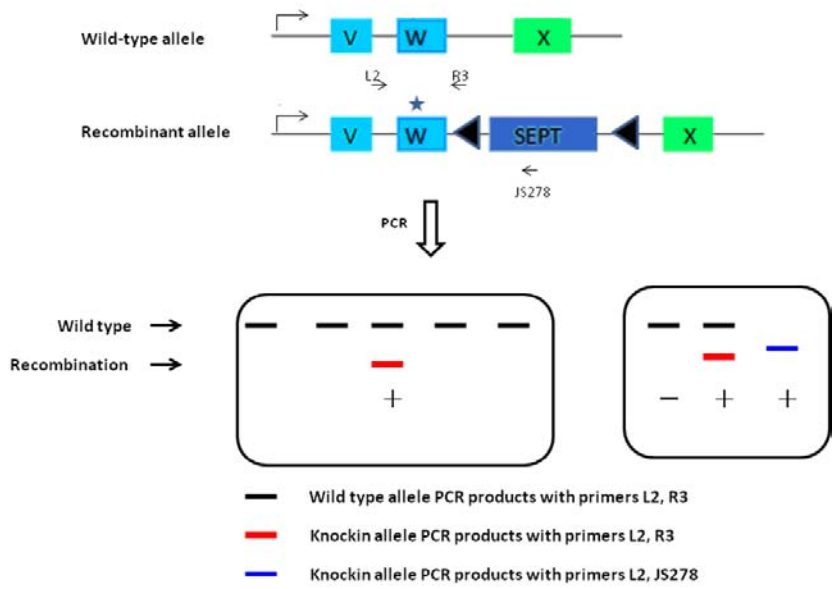

Figure 4: Screen and validation for homologous integration. A short sequence derived from the locus of interest is included in a primer used to synthesize an LHA. PCR amplification using a primer that anneals to this endogenous sequence (Primer R3) is paired with the outside primer (Primer L2). Both wild-type and knock-in alleles yield signals, as assessed by agarose gel electrophoresis; however, the signal derived from the knock-in allele is smaller and therefore more robust. Primers L2 and R3 were tested for generation of the expected amplicon from normal genomic DNA before vector construction. Validation is usually performed first through PCR with Primers L2 and JS278, one primer annealing within the SEPT element, then sequencing the PCR product.

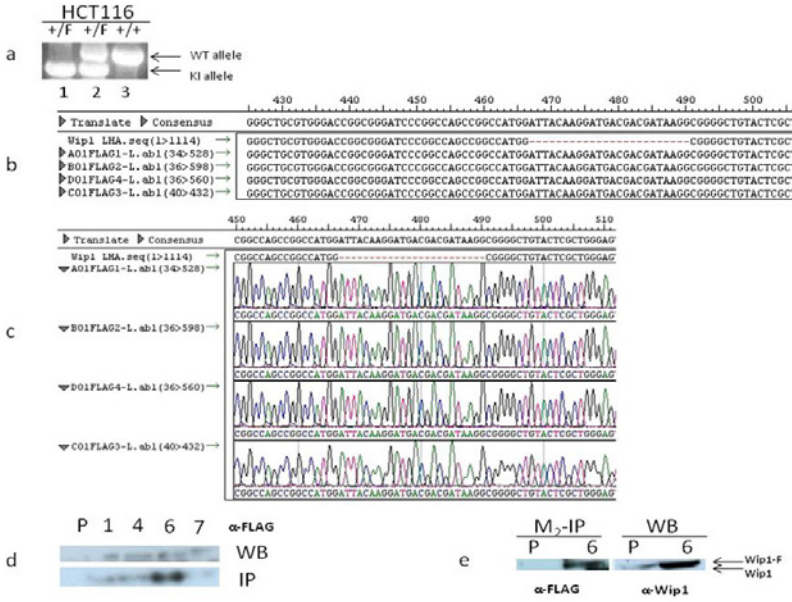

Figure 5: Validation of the *wip*1 FLAG knock-in allele. (a) PCR-based identification of FLAG-tagged *wip*1 alleles. +/+ refers to homozygosity for the unmodified parental allele, whereas +/F refers to derivative in which one allele has been modified by the addition of an in-frame amino-terminus FLAG epitope. Lane1: PCR product of genomic DNA of HCT116 *wip*1 FLAG knock-in cell strain with primers L2 and JS278 (Figure 4); Lane 2: PCR products of genomic DNA of HCT116 *wip*1 FLAG knock-in cell strain with primers L2 and R3 (Figure 4); Lane 3: PCR product of genomic DNA of parental HCT116 cell strain with primers L2 and R3. (b) Sequencing-based identification of FLAG-tagged *wip*1 alleles. PCR fragment in Lane 1 of (a) was firstly cloned into TOP10 vector. Four plasmids were extracted from four different single colonies and then sequenced with M13 forward primer. Correct FLAG encoding sequence was identified. (c) Sequencing results with four color fluorescence display. (d) Confirmation of FLAG-tagged alleles by western blot. The upper panel depicts a direct western blot using FLAG antibody on lysates from parental HCT116 cells (+/+) and four independently derived heterozygous (+/F) FLAG-*wip*1 epitope-tagged clones. The lower panel depicts a FALG IP/FLAG western blot from parental cells (+/+) and four independently derived FLAG-tagged derivatives (+/F). #6 cell strain was found to be optimal choice for the following experiment. (e) Further identification of #6 cell strain with western blot and immunoprecipitation. The left panel depicts total protein (2 mg) from parental HCT116 cells (+/+) and HCT116 FLAG KI #6 cells (+/F) were incubated with α-FLAG M2 agarose beads followed by α-FLAG antibody western blot. The right panel depicts a direct western blot using *wip*1 antibodies on lysates from parental HCT116 cells (+/+) and HCT116 FLAG KI #6 cells (+/F). The addition of a FLAG epitope increases the size of the encoded protein by ~1 kDa.

identical to those in another position within the targeted locus. The wild-type allele generates a PCR signal that is distinguishable from the positive (targeted) signal by its different size and thereby produces a positive control for each PCR in the screen.

After identification of a correctly targeted allele, part of the targeting construct is excised using *cre*. Cre can be introduced into the clone either by transfection of a mammalian expression vector or *via* infection with an adenovirus expression vector (Vector Biolabs). Following transfection or infection, clones are isolated by limited dilution and tested by Western blot with adenoviruses, more than a third of the resultant clones are generally found to have properly excised the sequences between the two LoxP sites.

A successful first round of gene targeting results in the generation of cell lines that are heterozygous with respect to the desired genetic alteration and sensitive to the drug initially used for selection (geneticin in the case of pSEPT-based approaches). For many purposes, a cell line that harbors a single targeted allele will be the end point. In cases when a homozygous cell line is desired, multiple alleles, and indeed multiple loci, can be targeted by iterative rounds of rAAV infection, drug selection and PCR screening.

Universal acceptor vectors for single-ligation assembly of AAV-based targeting vectors

One factor limiting the ease and speed of human somatic cell gene-targeting projects has been the technical challenges inherent to vector assembly. Several different approaches have been tried in an effort to simplify this process. For example, Todd and others have described approaches that exploit the high rate of homologous recombination in *S. Cerevisiae* to build targeting vectors with the needed junctions without the need for conveniently located restriction sites [14]. Kohli et al. have employed a nested PCR strategy for the creation of targeting vectors with only a single-ligation step [5]. However, these and other related strategies have had the disadvantages of either requiring multiple consecutive assembly steps and / or being insufficiently robust.In an effort to remedy these difficulties, universal AAV-2 acceptor vectors were created that makes it possible to perform vector assembly in a single ligation [7]. These acceptor vectors contain polylinkers with multiple unique, rare restriction sites flanking a FLOXed Neo^R gene, making it possible to subclone PCR-generated homology arms into the acceptor vector simultaneously.

The acceptor vector for *wip*1 FLAG KI contained a SEPT-*neo* gene for creation of promoter trap targeting vector. Of note, the SEPT cassette contains a splice acceptor followed by an IRES-*neo* and was described by Topaloglu et al [6].

A general approach for the creation of human epitope-tagged vectors and its application to *wip*1

This new acceptor vectors made it theoretically possible to build targeting vectors in a single-ligation step. To do this, left and right homologous arms were created by PCR from a human HCT116 cell genomic DNA template, and then simultaneously cloned into the polylinkers that had been built into the acceptor vectors. This was successfully performed for *wip*1 as described in Materials and Methods section and depicted in (Figures 2 and 3). Importantly, this ligation step was extremely robust, and the expected recombinant plasmid was present in 50% of all tested bacterial colonies.

Since the left arm of the targeting vector was designed to contain the initiating methionine codon, it was possible to add an epitope tag to the sequences encoding the amino-terminus of the targeted protein. Of present known epitopes, Myc, FLAG has been confirmed the best combination of efficiency and specificity for immunoprecipitation [7]. As such, FLAG epitope was employed in the epitope-tagging vectors described herein. The details of the mutagenesis reaction for insertion of FLAG are described in Materials and Methods section, and the scheme is depicted in Figure 1b.

Creation of human HCT116 cells with endogenous FLAG-tagged alleles of *wip*1

Once the *wip*1 FLAG-tagging vector had been created, it was packaged into AAV virions, which were used to infect human colon cancer HCT116 cells. Individual G418-resistant colonies were obtained and tested by PCR for the presence of homologous integration of the FLAG-tagging vector (Figure 4 and Figure 5a). PCR sequencing demonstrated that all the PCR-positive clones had undergone the desired modification in which a FLAG tag had been inserted in-frame immediately after the initiating methionine of the endogenous *wip*1 gene (Figures 5b and 5c). Positive clones were infected with a Cre-expressing adenovirus as described in Materials and Methods section to remove the FLOXed Neo^R gene and restore the targeted *wip*1 allele to its natural configuration.

Validation of human HCT116 cells with FLAG-tagged *wip*1 gene

We picked 4 candidates HCT116 *wip*1 FLAG KI cell line, numbered as HCT 116 *wip*1 FLAG KI #1, 4, 6 and 7, respectively, in which the endogenous *wip*1 gene had been modified *via* the addition of an amino-terminus FLAG, they were then validated by immunoprecipitation and western blot with antibodies to FLAG and WIP1. IP/western blots performed with FLAG antibodies demonstrated the presence of FLAG-WIP1 protein in FLAG-tagged HCT116 cells but not in parental cells (Figure 5d). Further, #6 cell line is the optimal choice for the following experiments. Similarly, as depicted in (Figure 5e), parental HCT116 cells had a single molecular weight band of WIP1 protein, whereas heterozygous FLAG-tagged cells had two molecular weight bands- the endogenous protein, and slightly larger protein reflecting the increased molecular weight caused by the addition of the FLAG tag (Figure 5e).

Discussion

Ectopic over-expression and RNAi knockdown have been widely used for functional research of human *wip*1 gene, while two-yeast hybrid and Co-IP have been used for screening its potential interacting proteins. However, these techniques have many shortcomings including too many false positives and biased selections. The stable cell lines harboring endogenous FLAG-tagged genes are good choice to overcome these flaws. Furthermore, endogenous FLAG tag not only facilitates purification of endogenous proteins and identification of novel protein complexes, but also validates novel protein-protein interactions discovered through other approaches when sensitive and specific antibodies to the components of the complex are unavailable. Of note, such stable cell lines may speed up the purification of medically useful therapeutic proteins at large scale under situations where ectopically expressed proteins are either inactive or insufficiently active. In addition, knock-in genome

editing technique described here can also be used to knock out those genes whose down-regulations will not influence the growth of their host cells by knock-in stop codon (TAA, TAG, TGA) just after targeted gene's start codon (ATG). Compared with Zhang et al.'s approach [15], the FLAG tag was prebuilt into the acceptor vector and FLAG knock-in vectors was created only by a single step in this study. Furthermore, the vector system here provides the choice of using the promoter trap architecture, resulting in substantially higher efficiencies of targeted integration.

However, for those genes whose exon 1 is too large for reverse primer to fall in intron 1, the method in this study may not be successful. Under this condition, in-frame tag FLAG just before stop codon in the carboxyl terminus of targeted gene can be tried. The next generation of FLAG-tagging vector systems may combine advantages of these different approaches, and dual- or multiple- tag systems are under way to avoid the contaminants common to single tag purification [7]. However, the conformation-altering effects may be unavoidable and should be evaluated carefully.

Recent years, Zinc Finger Nucleases (ZFN), Transcription Activator-Like Effector Nucleases (TALEN), and Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/ Cas9 system have been reported to successfully construct knock-out/-in cell lines and / or animals *via* genome editing [16], of which CRISPR/Cas9 system seems robust and popular [17], however, off-target effect is their common drawbacks [18], needing further efforts to exclude off-targets. Compared with these new favorites, relatively classical method described here is still worthwhile to be shared with peers as a selectable choice. Its combination with CRISPR/Cas9 may not only provide great broad platforms for genes' function investigation, but also a valuable means to treat many diseases from Mendelian disorders to cancers, and will revolutionize medical cares for many complex genetic diseases in the future as well [19,20].

In summary, an approach to in-frame FLAG-tag an endogenous gene *wip*1 in human HCT116 cells was described, and a novel HCT116 cell line with *wip*1 FLAG KI was successfully established in this report. Recently, WIP1 phosphatase was reported to play important roles in cancer [11], immunity [12] and aging process [11]. Further implementation and improvement of this technique will make it possible to find many novel WIP1- interacting proteins during human carcinogenesis, immunity and aging.

Conclusion

A novel HCT116 cell line with FLAG Knock-in in the 5' terminus of its *wip*1 gene was constructed and validated. The cell line will facilitate screening potential proteins interacting with WIP1 and constructing the corresponding signaling pathways in carcinogenesis, immunity and senescence.

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