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Development of CBX5 Knockout HeLa Cells by CRISPR/Cas9-Mediated Genome Editing

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ABSTRACT

CRISPR/Cas9 technology has gained immense popularity in last decade due to its ability to knockout gene of interest in target cells. In the present study, recombinant pL.CRISPR.EFS.GFP vector having five pairs of gRNA designed against CBX5 gene was generated. When it was transfected in HeLa cell line, the vector successfully knocked out the target gene. The knockout was preliminary confirmed by PCR-based amplification of region of interest. The sangar sequencing revealed the precise deletion of 369 bp from CBX5 gene rendering it "shut down" completely. Despite the knockout of this gene, no significant changes in cell morphologies were observed when compared with WT HeLa cells which demonstrated the dispensable nature of the gene for HeLa cell line. Furthermore, the detailed methodology for knocking out the CBX5 gene has been provided.

Key words: CRISPR/Cas9, knockout, CBX5, gRNA, HeLa

INTRODUCTION

Clustered regularly interspaced short palindromic repeats (CRISPR)-Cas system is a powerful genome editing technique with a tremendous potential for medical applications (Rodríguez-Rodríguez *et al.*, 2019). First found in bacteria as a component of a versatile immune defense against bacteriophages, CRISPR/Cas9 complex targets and cleave viral DNA rendering the invading viruses disabled (Höijer *et al.*, 2022). The CRISPR genome altering tool, comprises of the CRISPR associated protein Cas9 isolated from Streptococcus pyogenes (SpCas9), is a RNA-guided nuclease that is coordinated to a particular genomic area by a single guide RNA (sgRNA). After successful recognition by the associated sgRNA, the Cas9 protein identifies the PAM (protospacer adjacent motif) next to the target sequence. This identification of PAM sequence is followed by the double strand break (DSB) by the Cas9 protein. This technique has been widely used to generate knockout cells since the DSB in host genome is followed by NHEJ (non-homologous end joining) repair mechanism which often leads to the loss of nucleotides and result in the loss of amino acids and/or in the frame shift mutations in the target protein. Therefore, the CRISPR/Cas9 mediated targeting of host genes often results in the shutting down of particular genes and resulting in the generation of knockout cell lines.

Ironically, the CRISPR/Cas9 system which has been developed by bacterial hosts as a defense system against the viruses, can also been employed to generate the KO cells lines to boost the production of viruses. It is well known that viruses rely critically on their hosts for replication. There are various pro-viral key host factors which are critically required for virus replications. Knockout of these critical pro-viral host factors have been shown to significantly suppress the viral replication (Chander et al., 2022). In response to the viral infection, host cells trigger various antiviral signalling to combat and suppress viral replication. In this regard, many antiviral genes have been identified for different

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viruses. Therefore, it is also plausible to boost virus production by generating cell lines devoid of host antiviral genes.

Within a short period of time after its discovery, the CRISPR/Cas-9 genome editing technique has already started impacting significantly in various fields, including healthcare, agriculture and biotechnology (Bao *et al.*, 2019; Jiang *et al.*, 2019; Chen *et al.*, 2020; Li *et al.*, 2020). Apart from Cas9, many other Cas proteins with diverse functions have been identified which broaden the applicability of CRISPR/Cas system (Liu *et al.*, 2020). As a result, understanding the functional applicability of the CRISPR/Cas system and the effective creation of knockout cells have now begun to be seen as pre-requisites for next-generation application in diverse biological fields.

In the present study, a Chromobox protein homolog 5 (CBX5) knockout HeLa cell line via CRISPR/Cas9 technology have been generated. CBX5 is a highly conserved, non-histone protein and perform many functions. Through interactions with crucial kinetochore, the encoded product is involved in the development of a functioning kinetochore (Strom *et al.*, 2021). The successful generation of knockout cell line was validated by polymerase chain reaction (PCR) assay and sangar sequencing. The methodology employed in the study has been described in detail in this article.

MATERIALS AND METHODS

HeLa (Henerietta Lacka) cell line was available in the lab and was grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with antibiotics and 10% fetal calf serum (FBS). pL.CRISPR.EFS.GFP was a kind gift from Dr Hinh Ly, University of Minnesota, USA. Antibiotics, Luria Bertani agar, Luria Bertani broth SoC media were procured from Sigma Aldrich. sgRNA were also procured from Sigma Aldrich. Restriction enzymes were procured from NEB (New England Biosciences).

The complimentary DNA sequence (CDS) of human CBX5 gene was downloaded from NCBI website. To retrieved sequence was subjected to online tool CRISPICK (*https://portals.broadinstitute.org/gppx/crispick/public*) for identification of potential sgRNA sites adjacent to PAM sequences (Li *et al.*, 2020) within the sequence. These sites may reside on sense or antisense strand of the DNA. The server shortlist potential sgRNA sequences for both sense and antisense strand. As shown in Table 1, four such sequences were chosen for sgRNA preparation. When designing the sgRNA oligos 20 nucleotides proceeding to PAM sequences are required while PAM sequences should not to be incorporate in the oligos. For each target, two oligos (forward and reverse), complementary to each other were designed. Besides these core 20 nucleotides, the restriction site overhangs complimentary to BsmBI digested pL.CRISPR.EFS.GFP plasmid were also required. Additionally, "G" was also required for the Polymerase III promoter for effective transcription of the sgRNA. If the first nucleotide at 5'-end was not "G" then it must be added while designing sgRNA. The selected sgRNA integrated all of these nucleotides in the way as illustrated below.

For sense strand

Forward sgRNA: 5'-CACC**G**N_{20}-3'

Reverse sgRNA: 5'-AAAC (Reverse complement of N_{20}) **C**-3'

For antisense strand

Forward sgRNA: 5'-CAAC**G** (Reverse complement of N_{20})-3'

Reverse sgRNA: 5'-AAACN_{20}**C**-3'

Table 1. gRNA sequences against CBX5 gene

Name of oligo	Sequences (5'-3')	Orientation
NVT116.CBX5-1F:	5'- CACCGGAATATCTACTGAAGTGGAA -3'	Sense
NVT117.CBX5-1R:	5'- AAACTTCCACTTCAGTAGATATTCC -3'	
NVT118.CBX5-2F:	5'- CACCG CAGAGCAATGATATCGCTCG -3'	Sense
NVT119. CBX5-2R:	5'- AAACCGAGCGATATCATTGCTCTGC -3'	
NVT120. CBX5-3F:	5'- CACCG AGAAGTCAGAAAGTAACAAG -3'	Sense
NVT121. CBX5-3R:	5'- AAACCTTGTTACTTTCTGACTTCTC -3'	
NVT122. CBX5-4F:	5'- CACCGTGAGCTAATTTCTGAATTTA -3'	Antisense
NVT123. CBX5-4R:	5'- AAAC TAAATTCAGAAATTAGCTCAC -3'	
NVT124. CBX5-5F:	5'- CACCGGAAGGTGCTAGACAGGCGCG -3'	Sense
NVT125. CBX5-5R:	5'- AAACCGCGCCTGTCTAGCACCTTCC -3'	

Generation of Plasmid Constructs Expressing sgRNA/Cas9 Complex

pL.CRISPR.EFS.GFP was a kind gift from Dr Hinh Ly, University of Minnesota, USA. It expresses Cas9 endonuclease, tracrRNA scaffold and GFP and has *BsmB*I restriction site for cloning crRNA-encoding nucleotide sequences under U6 promoter. Twenty nucleotide long sgRNAs-encoding sequences (Table 1) were cloned into pL.CRISPR.EFS.GFP at *BsmB*I site. *CBX5* that is known to play anti-HSV1 function was targeted at four different sites in HeLa cells.

Annealing of Oligos and Cloning them at BsmBI Site of Plasmid

First, the pL.CRISPR.EFS.GFP vector was digested with BsmBI restriction enzyme according to the manufacturer's protocol followed by gel purification. The concentrations after gel purification fell in the range of 100-500 ng/µl. Next, the oligos (100 pmol stock concentration) were annealed to each other using following reaction:

- Anealing of oligos:
  oligo1 (forward): 7.5 µl (about 1µg total)
  oligo2 (reverse): 7.5 µl
  10X Annealing buffer*: 5 µl
  H_2O: 30 µl (total 50 µl volume)

Reaction condition – 95°C for 10 min, immediately on 70°C water till it reached at RT. Stored at -20°C

**(10X annealing buffer: (100 mM Tris pH 7.5-8.0, 1M NaCl, 10 mM EDTA)*

After successful annealing, the next step was to phosphorylate the annealed oligos using following reaction:

- Phosphorylation of annealed ologos:
  Annealed oligos: 20 µl (about 20 µg/ul)
  T4 PNK: 1 µl
  10X ligase buffer: 2.5 µl
  H_2O: 1.5 µl (25 µl volume)

Reaction condition – 1 h at 37°C

Note – Dilute phosphorylated oligos 1:1 with nuclease free water (final concentration of oligos was nearly 10 ng/µl at this stage)

Phosphorylated and annealed oligos can be ligated with the vector at BsmBI site using following reaction:

- Ligation:
  BsmbI digested pL.CRISPER.EFS.GFP: 1 µl
  10X ligase buffer: 0.625 µl (7.5 µl insert already containing 10X buffer)
  Insert (Annealed oligos): 7.5 µl (about 100 ng total)
  T4 DNA ligase: 1.5 µl (25 µl volume)

Reaction condition : The ligation reaction should be done at 4°C overnight.

After successful ligation, the next step was to transform the competent NEB stable cells (NEB Biosciences, UK) according to the manufacturer's protocol. Briefly, the 5 µl ligated product was incubated with 30 µl NEB stable cells for 30 min on ice followed by 1 min heat shock at 42°C using water bath. This was followed by again incubating them on ice for 1-2 min. After transformation, the cells were grown in 1 ml SoC media in 37°C BOD shaker incubator at 150 rpm for at least 2 h.

Since, the vector has ampicillin resistance gene, the cultured bacteria were plated on Ampicillin (100 µg/ml) plates and incubated overnight. Next day, the transformed bacterial colonies appeared on the ampicillin plate. Around 4-5 single cell colonies per petri dish were transferred in 8-10 ml LB broth containing ampicillin 100 µg/ml followed by overnight incubation at 37°C and 150 rpm. Post incubation, around 1 ml of bacterial suspension was preserved using 30% glycerol (v/v) and stored at -80 °C. For validation purpose the rest of the bacterial culture was subjected to plasmid isolation using QIAGEN mini plasmid isolation kit followed by restriction enzyme digestion using appropriate enzymes. For example the PstI restriction enzyme digested the pL.CRISPR.EFS.GFP vector in the following fashion. After successful validation, the preserved clones were amplified in 100 ml LB agar supplemented with ampicillin in similar way described before. The suspensions were subjected to plasmid isolation using QIAGEN midi plasmid isolation kit according to the manufacturer's protocol. The isolated plasmid having concentration >1 µg/µl was used for transfection. Four plasmids construct that express sgRNAs targeting at four distinct sites

within CBX5 gene were transfected. Cells were simultaneously transfected (according to manufactuer's protocol) with 3 µg of each of four plasmids in a 25 cm^2 cell culture flask of HeLa cells using Lipofectamine 3000 and observed for the expression of GFP under microscope. The transfected cells usually gave fluorescence 24-36 h after which they were sorted by FACS (Fig. 1) at Translational and Health Science Technology Institute (THSTI), Faridabad, India. The sorted cells were cultured by limiting dilution assay in 96 well cell culture plates to get final seeding density of a single cell/well. The cells were observed daily for appearance of colonies. Wells with a single colony were selected for further propagation (scale up). After achieving sufficient number of cells, usually a confluent 25 cm^2 flask, and cells were analyzed for editing of the targeted genome.

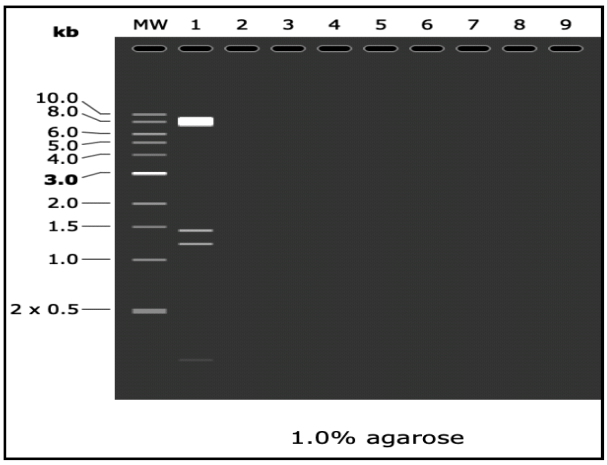

Fig. 1. 1% agarose gel run of pL.CRISPR.EFS.GFP vector digested with PstI restriction enzyme. Ladder - 1 Kb.

Knockout validation: CRISPR induced indels were validated by amplification and nucleotide sequencing of targeted region of gene (Jin *et al.*, 2020). For CBX5 gene knockout validation in HeLa cells, first the primers were designed to cover the gRNA sites of the gene. Next, the cells were harvested from confluent T25 cm2 flask (both from wild type and knockout HeLa cells) and subjected to RNA isolation using TRIazol method. The isolated RNA were then subjected to the cDNA preparation followed by PCR reaction. The resultant amplified gene fragments were viewed in 1% agarose gel. After successful preliminary validation of gene knockout the PCR products were sent for sangar sequencing to further confirm the number of nucleotide deletion from target gene.

RESULTS AND DISCUSSION

Five gRNAs using CRISPICK online tool against CBX5 genes were designed (Table 1). After successful annealing, phosphorylation, ligation and transformation successfully generated the recombinant pL.CRISPR.EFS.GFP plasmid with gRNAs inserts. The complete map of pL.CRISPR.EFS.GFP having BsmBI site for gRNA insert is shown in Fig. 2.

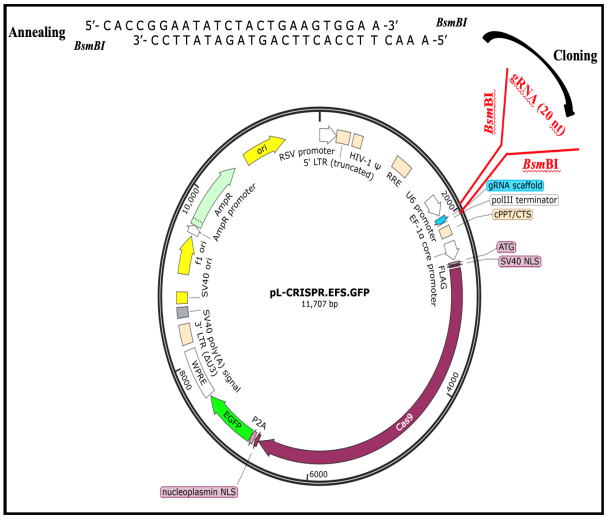

Fig. 2. pL-CRISPR.EFS.GFP plasmid map rendered using Snapgene software (version 4.3.6). The gRNA ligation with the vector is at BsmBI site.

After successful plasmid recombination, transfection was performed in HeLa cells for CBX5 knockout experiments. The GFP producing HeLa cells were sorted using Fluorescent Assisted Cell Sorting (FACS) and further diluted and seeded in 96 well plates to pick single cell clones (Fig. 3). The clones were further transferred in bigger flasks subsequently to get better confluences. The cells were then subjected to PCR validation.

1% agarose gel electrophoresis revealed that the band size of amplified product of CBX5 knockout HeLa cells was much smaller than the WT HeLa cells (Fig. 4a). Confluent monolayer of HeLa cells, in T25 cm^2 flask, was transfected with 25 µg of pL.CRISPR.EFS.GFP-*CBX5 (target gene)* plasmid construct. At 48 h post-transfection, cells were trypsinized and GFP producing cells were sorted. The sorted cells were cultured in 96-well tissue culture

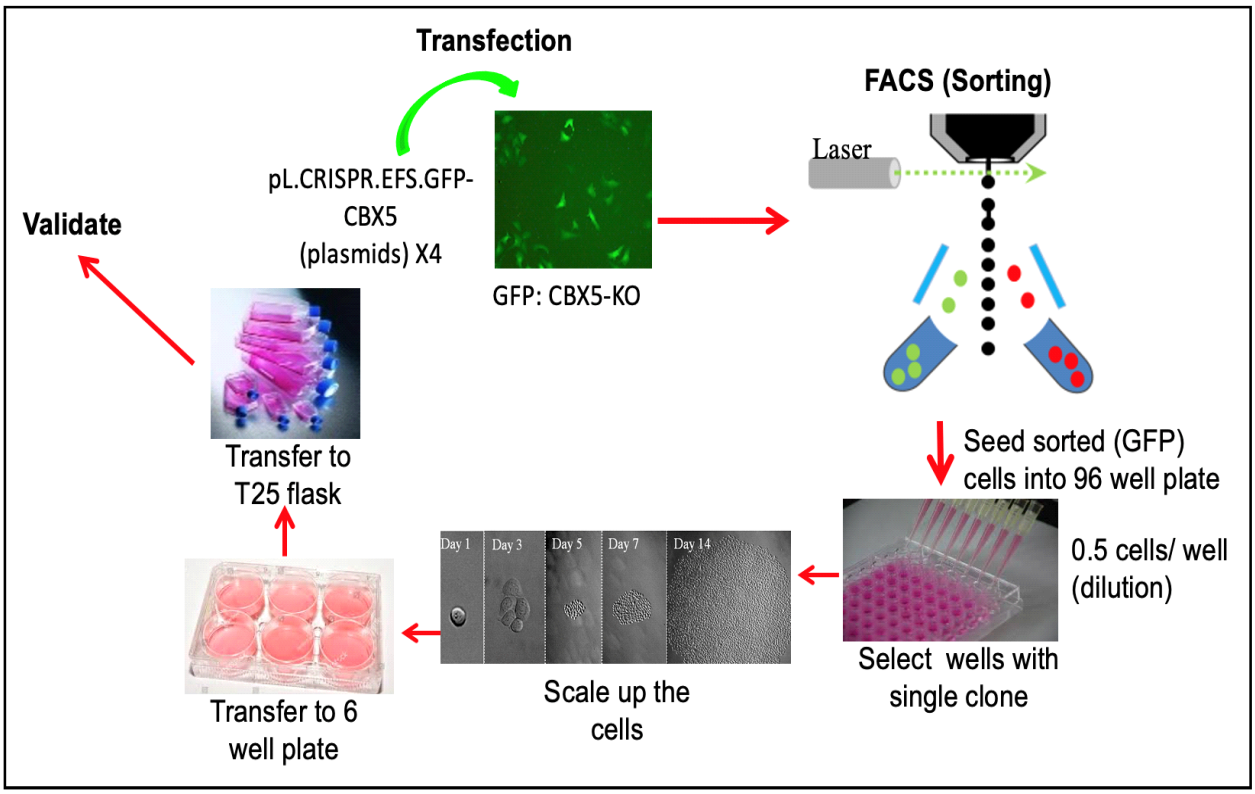

Fig. 3. Overview of generating knockout cells by CRISPR/Cas9-mediaetd genome editing.

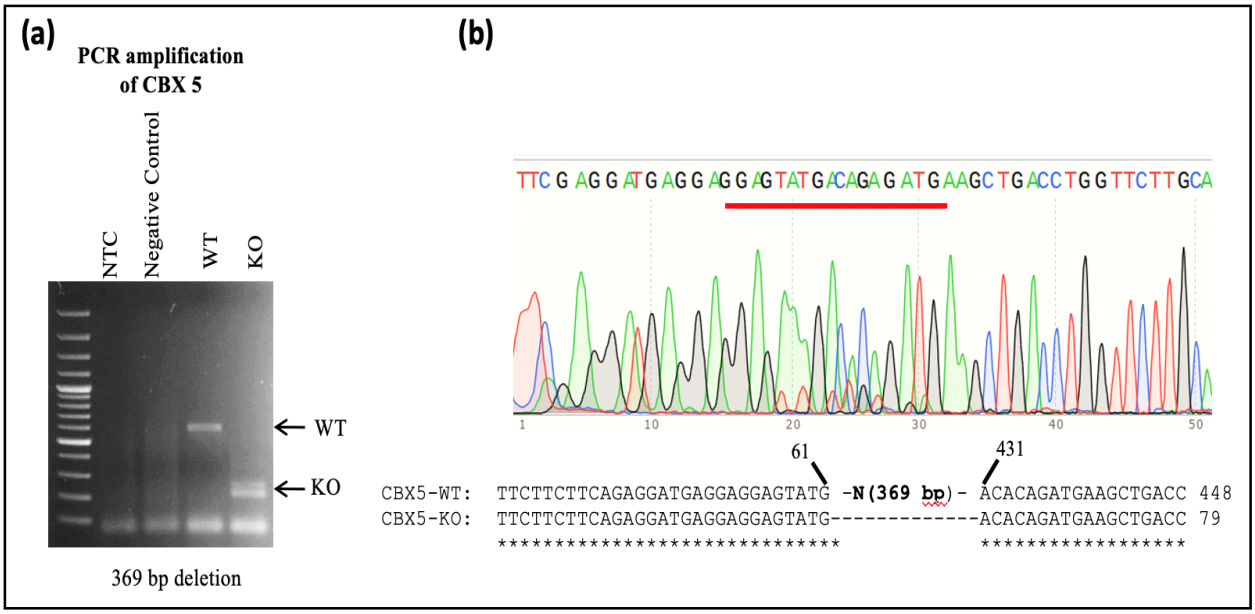

Fig. 4(a). 1% agarose gel electrophoresis of WT and CBX5 KO HeLa cells and (b) the nucleotide sequencing depicting deletion of 369 bp of CBX5 gene of HeLa KO cell line.

plates in a limiting dilution manner. The wells with single clones were selected and further cultured to scale up until obtaining confluent monolayers of one T25 flask. The targeted gene was amplified from all the selected clones by PCR. The clones with a shortened length were further confirmed by nucleotide sequencing.

This analysis gave good indication about the successful knockout of the target CBX5 gene in HeLa cells. To gain further insights and confirm the number of deleted nucleotides of CBX5 genes sangar sequencing of the PCR amplified products was performed (Fig. 4b). The sangar sequencing revealed a deletion of 369

bp fragment from CBX5 gene in knockout cell line which should be the result of DSB mediated NHEJ repair mechanism of HeLa cells since this repair mechanism was putatively error-prone and often resulted in base pair deletions of various lengths (Song *et al.,* 2021). After successful validation of gene knockout, clones were preserved using cryoprotectant and an aliquot was used to check any morphological changes in the cell line due to CBX5 knockout. No drastic morphological change was observed in knockout cell line when compared to WT cell line even after 10 passages. This indicated the dispensable nature of CBX5 gene in HeLa cells. Overall, the CBX5 knockout HeLa cell lines were successfully generated.

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