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Optimized guide RNA design and evaluation of CRISPR/Cas9 cleavage efficiency in LEP gene knockout

Nguyen Le Tram Anh[1], Au Duong Tuyet Mai[1], Nguyen Thi Thuong Huyen[2], Thai Ke Quan[3], Nguyen Dang Quan[*1]

[1]Biotechnology Center of Ho Chi Minh City (HCMBiotech), Highway 1 Trung My Tay Ward District 12 Ho Chi Minh City, Vietnam

[2]Department of Biology, HCMC University of Education, An Duong Vuong, Ward 4 District 5, Ho Chi Minh City, Vietnam

[3]Saigon University, An Duong Vuong, Ward 3 District 5, Ho Chi Minh City, Vietnam

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Abstract

Streptococcus pyogenes (SpCas9)-derived CRISPR/Cas9 system (SpCas9) is now a valuable tool for gene-editing technology, although their widespread applications is obstructed by a backwardness of knowledge about the activity of guide RNA (gRNA). A specific gRNA must maximize on-target (effective Cas protein guidance) while minimizing off-target sites. Balancing these two requirements is a challenging task. Therefore, the optimization of the sgRNA design is essential for the application of CRISPR/Cas9 towards knockout gene modeling, significantly lowering *prices and time-optimizing* to generate genetically modified animals. In this study, sgRNAs had been screened and validated *in silico* before inserted into the pX330 vectors *in vitro* validation. We randomly screened 468 single-stranded RNA sequences in the protein-coding regions of the *lep* gene. The sgRNAs were evaluated for their pairing in the target sequence region and validated for genomic random pairing activity. Next, we determined their activity on the pX330 plasmid by assessing the fluorescence expression in the HEK293 cell line. Of 468 screened sgRNAs, two sgRNA 28 and sgRNA 95 showed the most specific shear potential *in vitro* model. These sgRNAs will be checked further for production of CRISPR/Cas-generated *lep* KO mice as animal model for studying obesity and diabetes in the future.

*** Corresponding Author:** Nguyen Dang Quan ✉ ndquan.snn@tphcm.gov.vn

Introduction

The CRISPR/Cas (Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-Associated *proteins*) system has been becoming an advanced gene-editing tool that revolutionizes genome editing techniques (Deshpande, Vyas *et al.*, 2015). The Cas9 nuclease is currently selected in most specific DNA modification studies (Tycko, Myer *et al.*, 2016). The significant growth of the CRISPR/Cas systems are generated by the computational tools that facilitate test design and analysis of results (Sledzinski, Nowaczyk *et al.*, 2020). Moreover, using CRISPR/ Cas9 for the DNA cleavage studies helps to reduce cost, simplify operation, and achieve the high-efficiency (Hajiahmadi, Movahedi *et al.*, 2019, Sledzinski, Nowaczyk *et al.*, 2020). The CRISPR/Cas9 originally is an RNA-mediated genetic immune system that fights against genetic agents (phage, plasmids) in bacteria and archaea (Hsu, Lander *et al.*, 2014). The rapid development in genomic engineering is driven by an ecosystem of computational tools to facilitate test design and analysis of results (Sledzinski, Nowaczyk *et al.*, 2020). The main function of CRISPR/Cas computation software is applied to assisting for designing gRNAs by predicting their effectiveness (working on target) and acting off target (Sledzinski, Nowaczyk *et al.*, 2020).

The successfully designed gRNA must maximize instructional effectiveness while keeping a minimum potential non-target cutting (CRISPR/Cas9 specificity guided by gRNA) (Hajiahmadi, Movahedi *et al.*, 2019). Stabilizing these factors has been big challenges in recent years. Many computer tools have been developed to support the design the specific gRNA (Wilson, O'Brien *et al.*, 2018). These tools aid researchers to choose the best candidates by eliminating non-target sites that have low cutting efficiency and a high potentiality for off-target effects (Bradford and Perrin, 2019).

Studies of the obesity-related diabetes in mouse showed the link between the obesity status and nucleotide mutations on the lep gene (Roh, Lee *et al.*, 2018). Leptin gene is significantly expressed in fatty tissue producing a 167 amino acid chain which then turns into a 147 amino acid mature protein (LEP protein) after the signal peptide removal. This protein is produced by the LEP gene locating on chromosome 6qa3.3. The binding of this LEP to the hypothalamus cell helps produce the feeling of fullness. A point mutation appearing in the coding region of the lep gene tends to cause the frameshift resulting the presence of an termination codon (Kanasaki and Koya, 2011) encoding a defected LEP. Therefore, this is an important protein in the control of weight and regulation of energy homeostasis. Leptin gene mutation causes severe obesity and hypogonadism in the patient and has also been linked to the development of diabetes. Thus, modifications of *the lep* gene may provide an opportunity to generate obese and diabetic mouse models (Moon, Dalamaga *et al.*, 2013). According to the released gene information, *lep* contains three exons, i.e., exon 1 (1 - 28), exon 2 (8701 - 8875), and exon 3 (10603 - 13656). Further analysis of the recently released *lep* KO mouse model also showed that exon 3 is amenable to LEP protein translation. We confirmed the exon 3 as the target sequence for *lep* gene knockout.

The ob/ob mouse model is the mutant mouse on the leptin coding gene, often exhibited uncontrolled eating, overweight, insulin resistance and hyperglycemia (Kanasaki and Koya, 2011). The effects of the increasing incidence of obesity and diabetes around the world have made the need for research models to develop therapeutic therapies. Several animal models of obesity are based on both the diet and the genetic obesity pattern. Current animal models are often associated with mutations that affect the production and response to the hormone leptin. The production of diabetic mouse models is usually created by cross-breeding between animal models of substrate redundancy or crossbreeding based on different mouse genetic models. Male ob/ob rats can sometimes be reproduced on some genetic background and follow a limited diet and leptin supplement. Under the same conditions, ob/ob females typically reproduce much less effective and require daily leptin injections from pregnancy to weaning. This method is often expensive and requires a high consumption of leptin (Ellett, Evans *et al.*, 2009).

Therefore, the production of mouse models of genetic obesity and diabetes using the CRISPR/Cas9 technique helps overcome some disadvantages of traditional breeding methods (Hall, Cho *et al.*, 2018). In this study, we performed large-scale *in silico* screening in the *lep* gene, intending to investigate how the elements affect CRISPR-based gene editing. We discover that DSBs caused by CRISPR/Cas9 are corrected in an expectable or unrespectable manner, contingent on the location of target gene. Moreover, simultaneous choosing two sgRNAs is an effective strategy for generation *lep* KO mouse model by CRISPR-mediated desired changes.

Materials and methods

Design of sgRNAs to knock out homologues of the lep gene

sgRNAs were designed using CHOPCHOP web-tool (Labun, Montague *et al.*, 2016) and ranked by efficiency score based on algorithms developed by Doench (Doench, Fusi *et al.*, 2016). The best sgRNA candidates were chosen with following rules (Fig. 1).

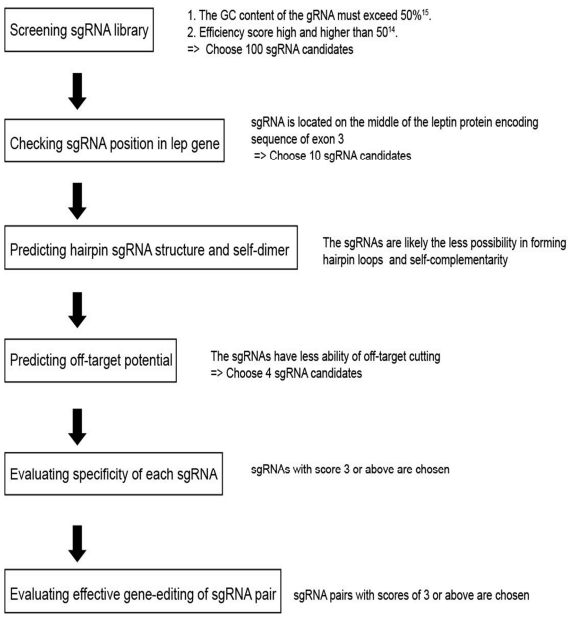


Fig. 1. Workflow for designing sgRNA to knockout lep gene by CRISPR/Cas9. The sgRNAs library was created to screen all sgRNAs based on the adjacent PAM sequence (NGG), sgRNA ranked based on different standards, then filtered to avoid factors that lead to off-target cleavage. Four sgRNA candidates have evaluated the efficacy of the CRISPR/Cas9 system on HEK293 cells.

Predicting off-target potential

Possible off-target matching *are* checked using Bowtie2 and IDT's CRISPR/Cas9 Design tool with rules outlined previously (Langmead, Trapnell *et al.*, 2009, Mashiko, Young *et al.*, 2014, Spiegler, Rath *et al.*, 2019, Sledzinski, Nowaczyk *et al.*, 2020).

Each of ten sgRNAs passed above rules was then mapped onto the mouse genome (mm10) for the off-target site matching (Langmead, Trapnell *et al.*, 2009, Mashiko, Young *et al.*, 2014, Sledzinski, Nowaczyk *et al.*, 2020).

Four candidate sgRNAs with the lowest mis-matching would be chosen for the in vitro validitation. Consequently, 4 sgRNA candidates were cloned based on the hSpCas9 and guide RNA expression vector.

Plasmid and gRNA preparation

The first vector is a plasmid pX330 (Addgene, #42230) expressing hSpCas9 and sgRNA was used to insert sgRNA sequence at the BbsI site (Cong, Ran *et al.*, 2013). The second plasmid (pCAG-EGxxFP, Addgen, #50716) has been included the overlapping of 482 bp EGFP encoding regions at the N and C ends that contains EGx and xFP fragments (Mashiko, Fujihara *et al.*, 2013).

This plasmid is seen as a reporter to evaluate the activity of the CRISPR/Cas9 system. The target sequence of exon 3 (420 bp) are amplified by PCR method and inserted into pCAG-EGxxFP vector at multiple replication sites (BamHI and EcoRI).

When the sgRNA linked pX330 plasmids and pCAG-EGxxFP-target (plasmid containing the third exon sequence of lep gene) transfected into HEK293 cells, the target sequence was disrupted by sgRNA guided Cas9 endonuclease. Then, the HDR (homology-dependent repair) was taken place.

Finally, the fluorescent signal was created by reconstituting EGFP expression cassette (Mashiko, Fujihara *et al.*, 2013, Mashiko, Young *et al.*, 2014) (Fig. 2).

HEK293 cell culture and transfection

HEK293 cells were cultured in the DMEM medium with 10% FBS supplemented. The plasmid mixture containing one microgram of pCAG-EGxxFP-target and 1 µg pX330-sgRNA is mixed with PEI in the ratio of 1:3 and incubated for 30 minutes.

The mixture is then gently dropped into each well. Finally, EGFP was observed at 48-hour, 72-hour time points after the insertion of plasmids into HEK293 (Mori, Yoshida *et al.*, 2020).

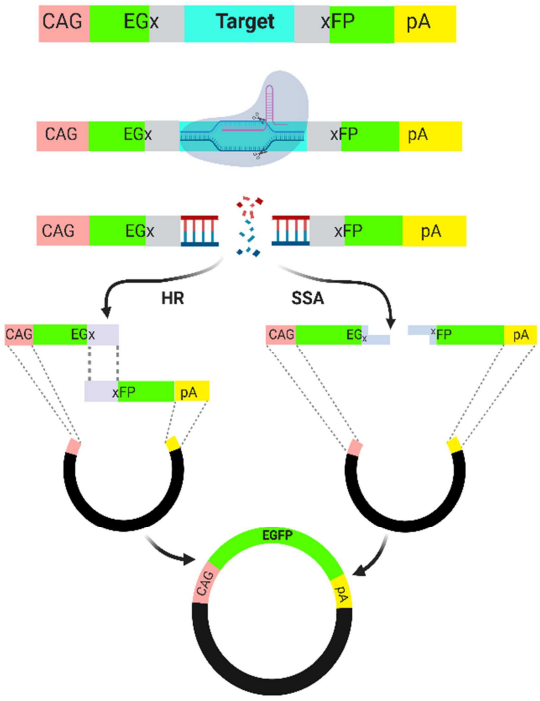


Fig. 2. Scheme of the EGFP expression cassette reconstitution.

Results

Sequence screening

Screening of sgRNA on the lep gene using CHOPCHOP showed 468 candidates which are 20 bp long followed by the PAM sequence (NGG). Of these, 100 sgRNA candidates with GC content greater than 50% and the efficiency score greater than 50 were chosen for further analysis (Table S2). Targeting on the exon 3 of the lep gene, sgRNA 7, 14, 20, 28, 66, 72, 95, 177, 181, 190 were chosen (table S3) while sgRNAs scattered over other regions of the lep gene were eliminated from the list.

Table 1. Predicted hairpin and self-complementarity structure of 10 sgRNAs.

sgRNA	Target sequence	Delta G of N20 sequence (kcal/mol)	Delta G of N40 sequence (kcal/mol)	Self-complementarity
7	GTCGGTATCCGCCAAGCAGA	-2	-4.6	0
14	GACTCTGGCAGTCTATCAAC	-2.3	-8.8	1
20	AGTCGGTATCCGCCAAGCAG	-2	-4.6	1
28	GATACCGACTGCTATGCAGG	-3.8	-7.5	2
66	TCGGAGATTCTCCAGGTCAT	-3.9	-6.5	1
72	GCTGCAGATAGCCAATGACC	-1.8	-5.2	2
95	GGAGGTCTCGGAGATTCTCC	-5.5	-9.8	3
177	GCCAGTGACCCTCTGCTTGG	-1.2	-8.8	0
181	CACCTCTGTGGAGTAGAGTG	-3	-6.4	4
190	GTCCAAGATGGACCAGACTC	-4.9	-8.3	4

Table 2. Prediction of CRISPR / Cas9 off-target cleavage in mm10 mouse genome.

sgRNA	Sequence	PAM	Gene	Locus
7	GTCCGCTTCCGCCAGGCAGA	CGG	NAT9	chr11:+115187372
	CTCAGTGTCTGCCAAGCAGA	GGG	4933429K18RIK	chr19:+45730333
	GTCTGTAGCCACCAACCAGA	AGG	CDHR2	chr13:-54713927
	GTGGGTCTCCTGCCAAGCAGA	CGG	BRINP1	chr4:+68841287
14	GAAGCTGTCAGTCTATAAAC	TGG	HMGCS1	chr13:-119707311
	GATCCTGGCAGTTTCTCAAC	CGG	ADGRB3	chr1:+25101485
	GACGCTGTCAGTCTATGAAA	AGG	ELMO1	chr13:-20608070
	GAAGCTGGCAGGCTCTCAAC	AGG	SHISA9	chr16:+11990205
	GACCCTGGGAGCCTAGCAAC	CGG	AGBL2	chr2:-90782720
20	AGCCGGTATCCGCCATGCTC	CGG	NOLC1	chr19:-46076025
	AGTCTGTAACAGCCAAGGAG	AGG	PRRG3	chrX:-71968497
	AGTCAGTATCTGCCAAGGAA	GGG	TRAF4	chr11:+78161465
28	GATTCCGAGTGCAATGGAGG	TGG	FER1L4	chr2:-156045440

sgRNA	Sequence	PAM	Gene	Locus
	GAGAACGACTGCTATTCAAG	AGG	GM35828	chr11:-105504828
	GAGGAGAGTCTCCAGGTTAT	TGG	FBXW2	chr2:+34822891
66	TCAGAGATTCCCCAGGTGAT	GGG	LIME1	chr2:+181381866
	TTAGAGGTTCTCCAGGACAT	GGG	IRAK3	chr10:-120182563
	TCGGAG-TGCTGCAGGTCAT	TGG	GM30067	chr2:-39111497
	ACTGCAGGTAGACAATGACC	AGG	RTP2	chr16:+23927236
	GCTGCAGAT-GCCAGAGACC	GGG	LOC108167911	chr11:+72767428
72	GCTGCAGCTAG-CAATGGCC	AGG	BAIAP2	chr11:+120005094
	GCTACAGCTCTCCAATGACC	TGG	CACNA1E	chr1:+154733153
	GCCGAAGATAGCCTATGACA	CGG	COG8	chr8:+107052925
	CGAGGTCCCGGGCATTCTCC	AGG	SACS	chr14:-61212208
	GGAGG-CACGGAGGTTCTCC	AGG	HELQ	chr5:+100798389
	GGAGGTCCCGCAGATGCTCC	AGG	ZFP92	chrX:-73422535
	GGAGGTCAGTCAGATTCTCC	TGG	INPP4A	chr1:-37369456
95	AGAGGTCTCGAAGGCTCTCC	AGG	INSRR	chr3:-87800524
	GGAGGTCACGGACATTCTCA	TGG	SLC30A7	chr3:-115990019
	GGAAGGCTCGGGAGATTCTCC	AGG	DHX8	chr11:-101766827
	GGAGGTCTCTGAGATCCTCA	TGG	DENND5A	chr7:-109921402
	GGAGGCTTCGGCGACTCTCC	TGG	TMEM246	chr4:-49587109
	TCCAGTGCCCCTCTGCGTGG	TGG	GM21885	chr11:-94280279
	CCCATTGACTCTCTGCTTGT	TGG	NLGN1	chr3:-26133634
177	TCCACTGGCCCTCAGCTTGG	GGG	LRCH3	chr16:+33010391
	GCAAGCGAGCATCTGCTTGG	AGG	GM33524	chr14:-70038942
	GCCACCGCCCCTCTGCTGGG	GGG	NACAD	chr11:+6605620
	GGCCTCTTTCGAGTAGAGTG	TGG	LOC102636795	chr6:-126772301
	CTCCTCAGGGAAGTAGAGTG	GGG	HMOX1	chr8:-75096931
181	CAACTCTGT-CAGTAGAGTG	AGG	HNRNPK	chr13:+58395232
	CAAGTCTGTGGAGT-GAGTG	TGG	PKHD1L1	chr15:+44535727
	GACCTCTGTGGGGTGGGGTG	GGG	IFFO2	chr4:+139617308
	GACCTAGCTGGATCAGACTC	TGG	CEACAM20	chr7:+19990143
	CTACAAGATCGCCCAGACTC	TGG	GM29854	chr16:+7771492
190	GTCCAAGCTGACCCAGCCTC	AGG	ELFN1	chr5:+139972309
	TTCCTAGATGGCCCAGAATC	TGG	A230050P20RIK	chr9:-20870834
	GAGCAAGATGGCGCAGACTC	AGG	HDAC1	chr4:-129542616

Checking of off-target sites

The best ten sgRNAs were subjected to Vienna RNA secondary structure server and Bowtie2 simultaneously to examine the hairpin loop formation, self-complement, and off-target cleavage. The sgRNAs 14, 72, 95, 177, 181, 190 showed high potentials of off-target cleavage with more than 5 matchings on different chromosomes. Notably, sgRNA 7 and sgRNA 20 are nearly similar with only one nucleotide sliding.

They shared the same possibility of secondary structure formation as well as the off-target matching. Therefore, only one of these two sgRNAs would be analysed further, and sgRNA 7 was randomly chosen. sgRNA 7, 28, 66 with low self-complementarity and low off-target sites were the best candidates for in vitro validation.

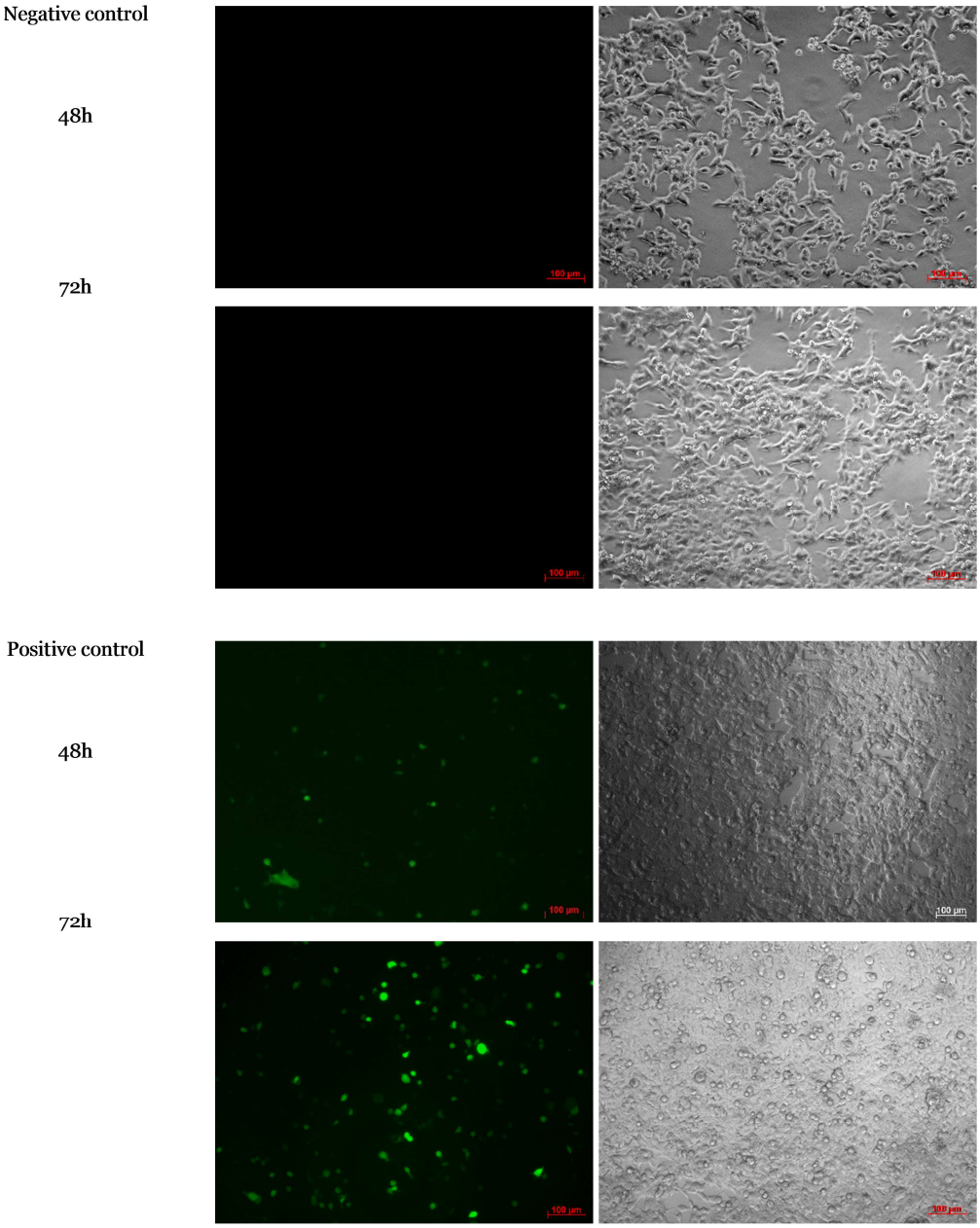
To improve the efficiency of CRISPR/Cas9 system, i.e the probability of lep protein breakdown, combination use of two sgRNAs were planned. The location of sgRNA 7, 28 on the 5' end and sgRNA 66 on the middle of exon 3 suggested that one sgRNA on the middle of exon 3 should be needed. Although sgRNA 95 was not good on the aspect of secondary structure formation, its location and the fact that it was one of the best ten candidates supported us in taking it into account to validate further. Finally, sgRNAs 7, 28, 66, 95 were synthesized and inserted into vectors for in vitro evaluation in HEK293 cells.

Construction and validation of CRISPR/Cas vector activity in vitro

We transfected simultaneously two vectors (pCAG-EGxxFP-target and pX330-sgRNA) to choose the best sgRNA pair by using the pCAG-EGxxFP-Cetn1 and pX330-Cetn1/1 as the positive standard of comparison (Fujihara and Ikawa, 2014, Mashiko, Young *et al.*, 2014, Mizuno, Dinh *et al.*, 2014). The EGFP signal was keep score with score 1: non-signal, score 2: lower signal than positive standard, score 3: equal control, score 4: higher signal than control (Mizuno, Dinh *et al.*, 2014). As the result of evaluation of cleavage efficiency, among the four pX330-sgRNA plasmids examined, sgRNA 7 had the highest EGFP signal, followed by sgRNA 28, 66 and 95 (Fig. 3 and Table 3).

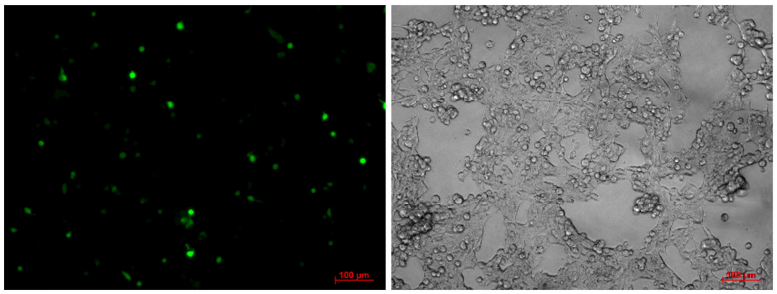
Table 3. Classification of the reconstituted EGFP fluorescence of HEK293 cells after co-transfection pX330-sgRNA and pCAG-EGxxFP-target plasmid.

sgRNA	Score
sgRNA 7	4
sgRNA 28	3
sgRNA 66	2
sgRNA 95	3
sgRNA 28 + sgRNA 66	4
sgRNA 28 + sgRNA 95	4
sgRNA 7 + sgRNA 66	2
sgRNA 7 + sgRNA 95	3



sgRNA 7

48h



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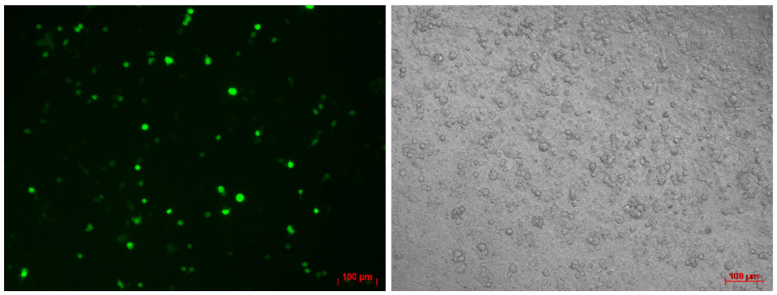

sgRNA 28

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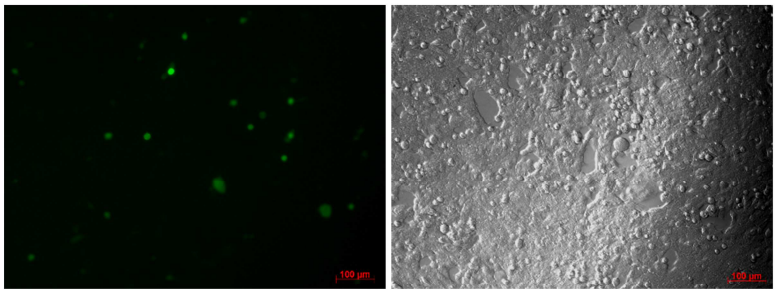

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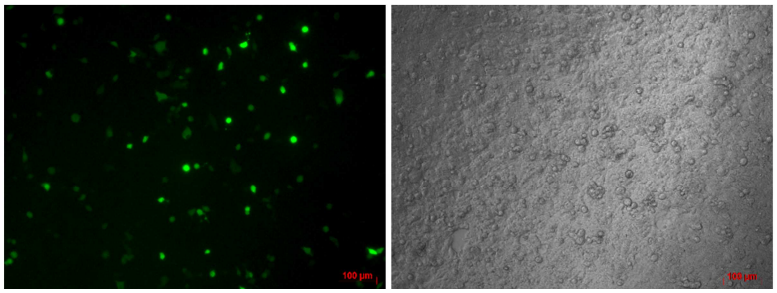

sgRNA 66

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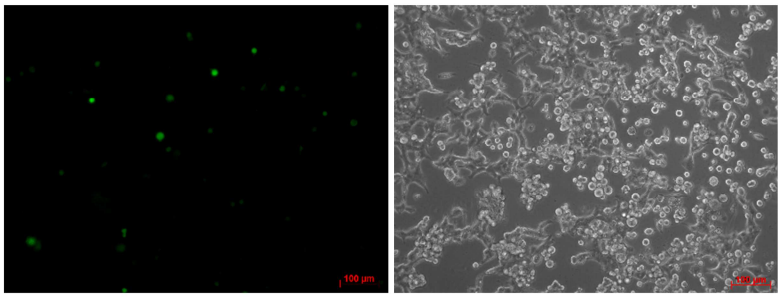

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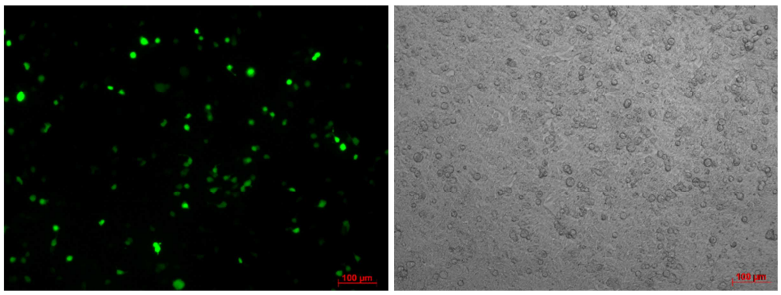


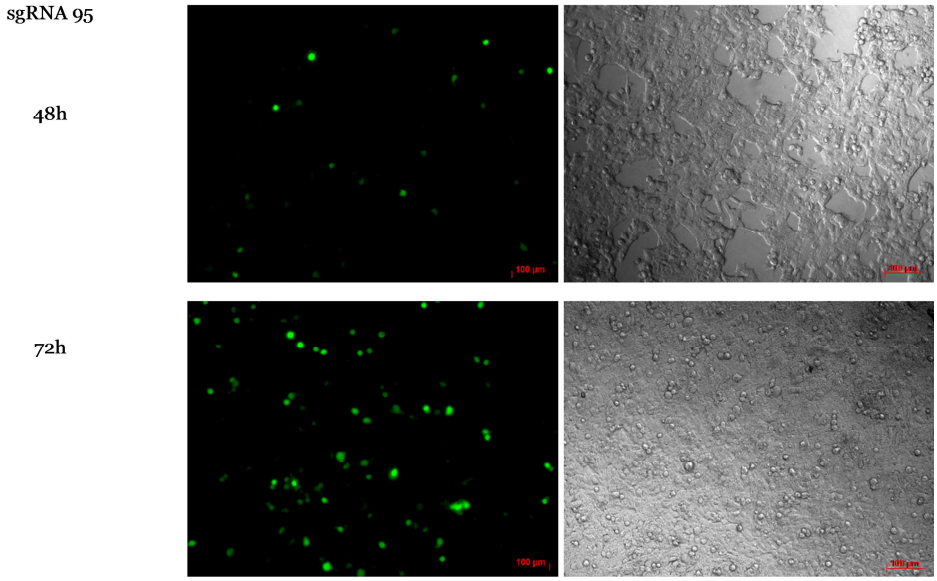
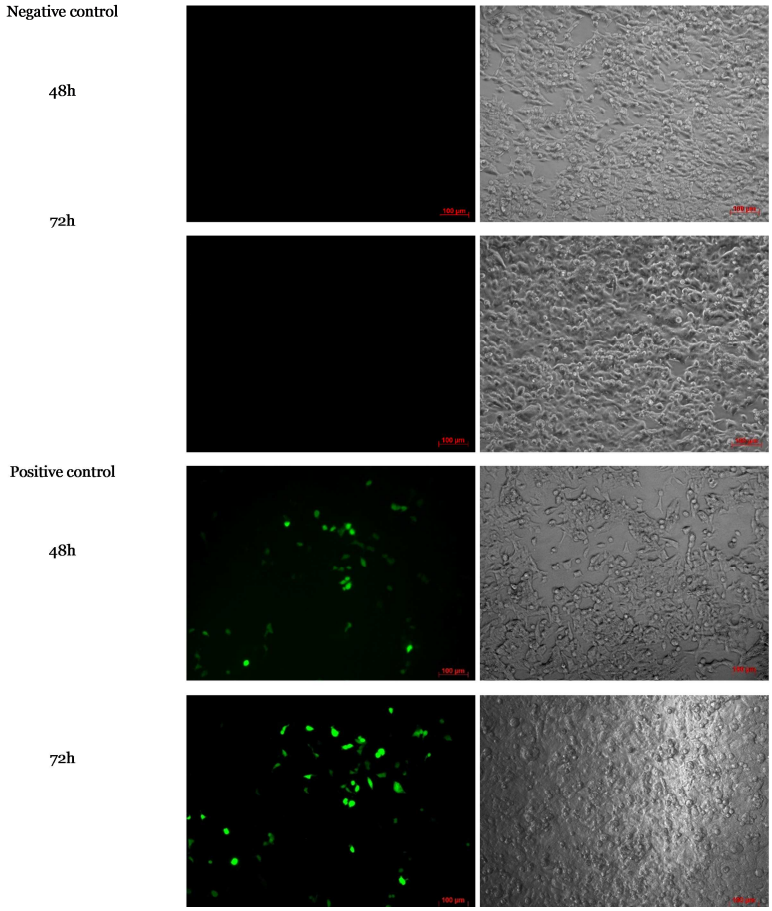


Fig. 3. Co-transfection the individual pX330-sgRNA plasmids.

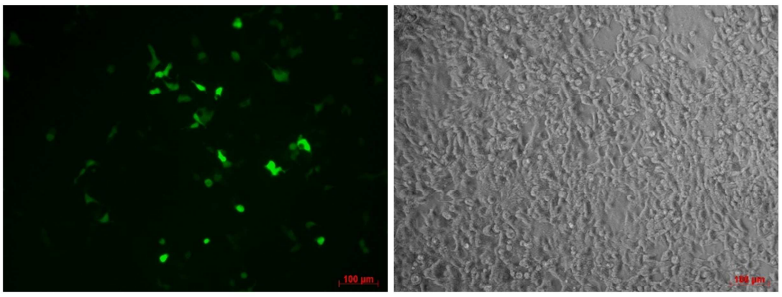
Effective genome editing of sgRNA pairs

Next, we co-transfected randomized these sgRNAs to choose a pair of sgRNAs for the best knockout efficiency (Fig. 4). Both sgRNA 28, 66 and sgRNA 28, 95 pairs give similar GFP signals (Table 3). However, the human U6 promoter required a guanine to be the transcription initiation site since this G initially affects positively the pX330 activity (Mashiko, Young *et al.*, 2014). Therefore, we believe that the efficiency of HDR-dependent precision genome editing of sgRNA 28 and sgRNA 95 is higher than the 466 screened sgRNAs.



sgRNA 28 + sgRNA 66

48h



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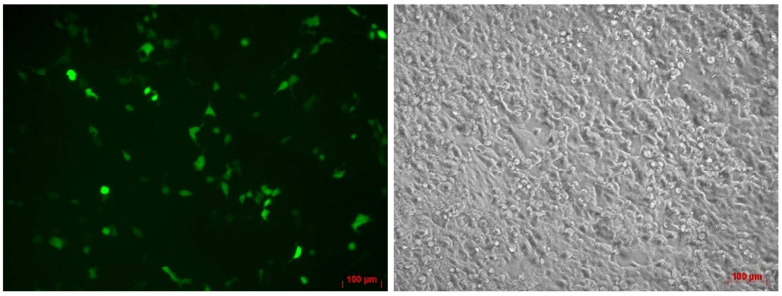

sgRNA 28 + sgRNA 95

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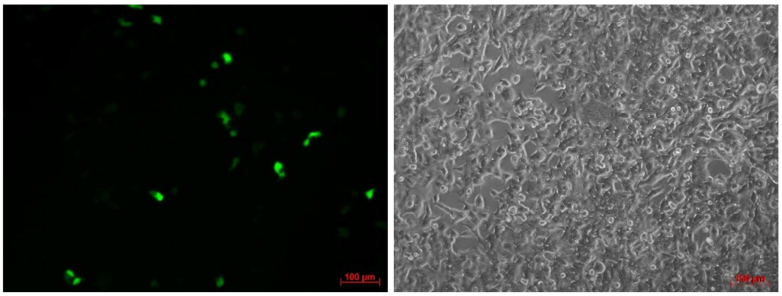

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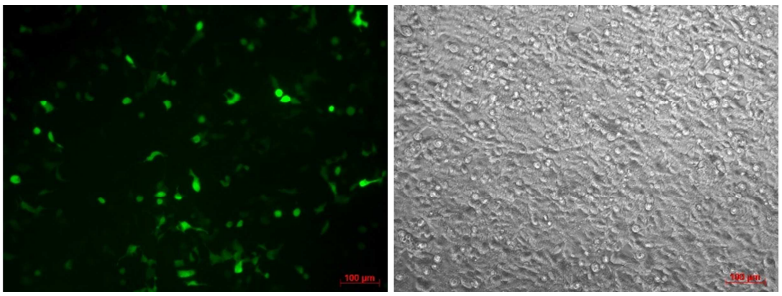

sgRNA 7 + sgRNA 66

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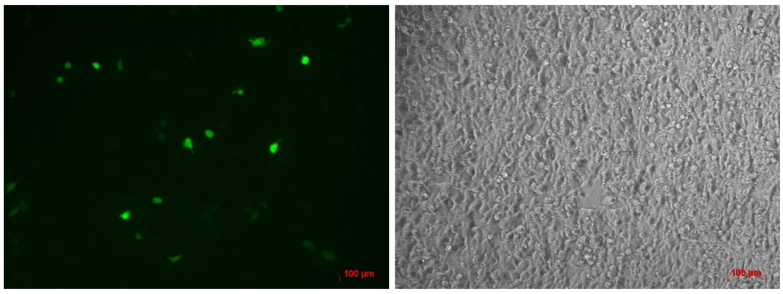

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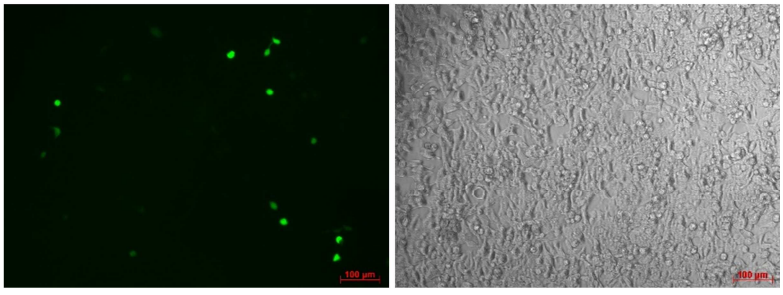

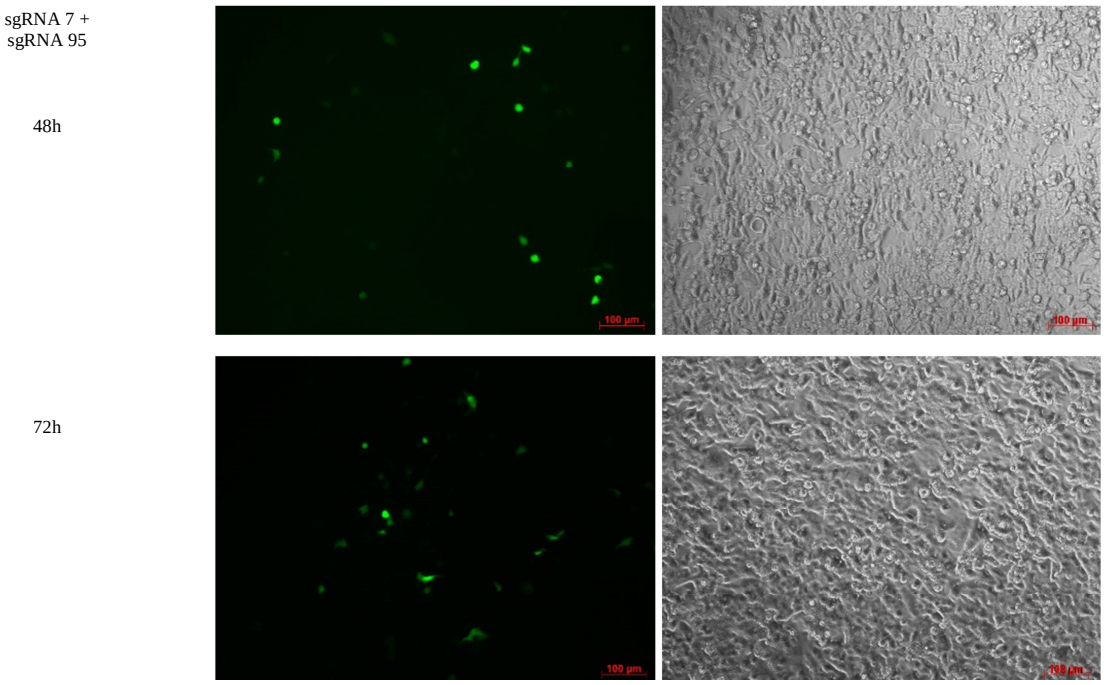

Fig. 4. Validating the efficiency of DSB mediated homology repair by utilizing 2 sgRNAs.

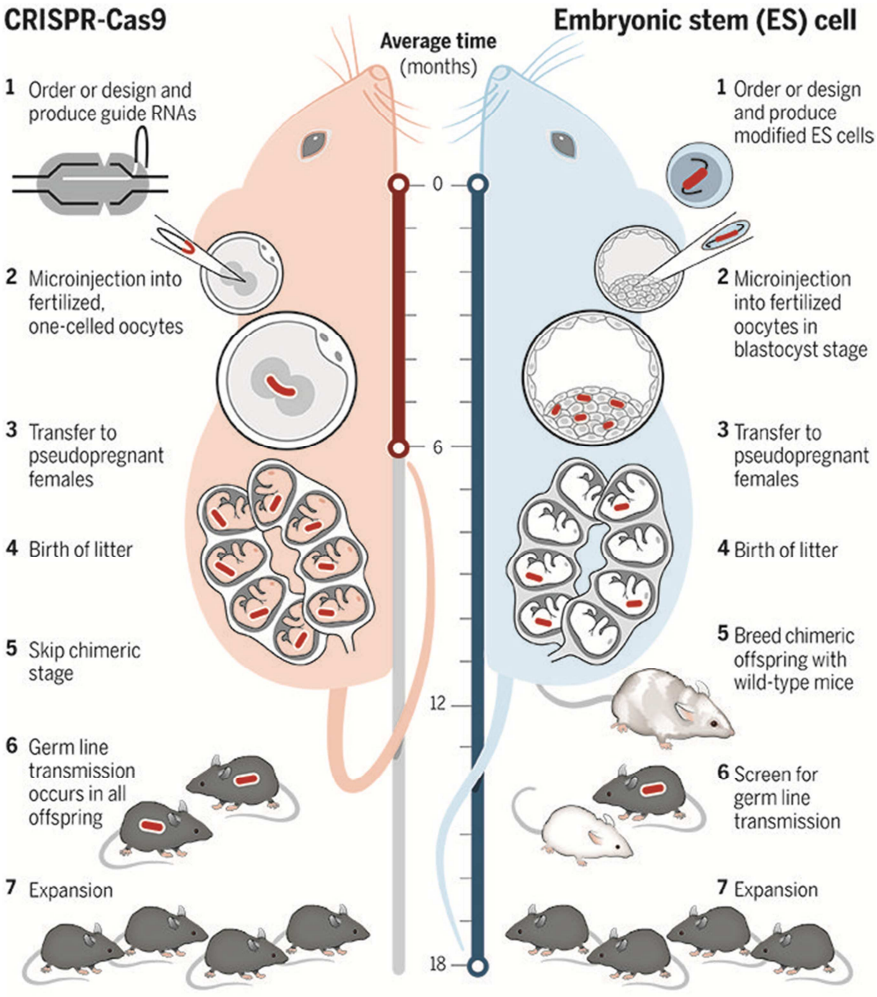

Fig. 5. Scheme of comparing the gene-editing technology using CRISPR/Cas and the approach of gene editing by embryonic stem cells.

Discussion

In the CRISPR/Cas9 system, results were ranked by: efficiency score and presence of guanin in target sgRNA sequence site. This score, developed by Doench and Hsu *et al*, ranges from 0 to 100; the higher the score indicates the more efficiency (Hsu, Scott *et al.*, 2013, Doench, Hartenian *et al.*, 2014). This score is considered as the probability of a particular gRNA that is able to be in the best cleavage activity group(Shalem, Sanjana *et al.*, 2014). Moreover, only five percent of gRNAs gain 50 points or higher in this scoring system. Recently, algorithms were advanced to predict on-target and non-target activities by Doench *et al* (Doench, Fusi *et al.*, 2016). Additionally, sgRNAs containing over-low or over-high GC content tended to be fewer effective activities (Doench, Hartenian *et al.*, 2014, Bortesi, Zhu *et al.*, 2016). Therefore GC content has a vital influence on the guide-efficiency of sgRNA (Bortesi, Zhu *et al.*, 2016). Following these rules, to make use of 10 sgRNAs, we screen for essential 468 sgRNAs in the *lep* gene.

Protein Cas9 must be combined with gRNA to convert an active Cas9-gRNA-DNA form to achieve site-specific DNA identification. Interestingly, Cas9 has a more direct association with stem-loop 1. In contrast, Cas9 has less extensive contacts to stem-loop 2, 3 in the sgRNA-linked Cas9 structure (Bruegmann, Deecke *et al.*, 2019). Many studies demonstrate that the sgRNAs lack binding the stem-loop 2, 3 are able to activate Cas9-based DNA split, though with less effectiveness. Stem-loop 1 is indispensable for the Cas9-sgRNA interaction. Because the destruction of stem-loop 1 removes completely, the cleavage of the Cas9-gRNA-DNA complex is able to inactivity (Jiang and Doudna, 2017, Bruegmann, Deecke *et al.*, 2019). We proceed with the gRNA designing that has less hairpin formation that causes the breakdown of stem-loop 1 to predict the hairpin loop of the first N20 and the 20 nucleotide initiation site of the tracrRNA to remove sgRNAs that damage the structure of stem-loop 1.

The main obstruction to the utilization of the CRISPR/Cas9 technique is non-target cleavage (Sung, Jin *et al.*, 2014). CRISPR/Cas9 is able to split unexpected gene sites and cause unintended mutations because the sgRNAs recognize the DNA sequence with some mismatch, known as unsatisfactory cleavage (Liu, Zhang *et al.*, 2020). Various attempts have been performed to decrease the non-target shear activity of Cas9-gRNA-DNA complex (Naeem, Majeed *et al.*, 2020). Non-target effects can be dramatically mitigated by prognosticating the specificity of the CRISPR/Cas9-based gene editing and creating optimal sgRNAs. To prognosticate the specificity of CRISPR/Cas9, two key approaches have been offered: (1) the association method, and (2) scoring method. The association method based on specialized algorithms, the gRNA is sorted by a particular genome after that the non-target positions and sequences will be outputted. This approach is chiefly utilized to discover all the non-targets in the *in silico* model. In the second method, sgRNAs are scored and ranked by utilizing defined rules to select the most specific for the trials (Liu, Zhang *et al.*, 2020). Accordingly, we evaluated self-complementary and non-target sites by mapping onto the whole mouse genome to choose 4 "functional" sgRNA for knockout of the *lep* gene.

We cloned four selected sgRNA sequences in pX330 vectors. These plasmids were co-transfected with the reporter plasmid into the HEK293. When utilizing the pX330-Cetn1 positive control, four pX330-sgRNA vectors showed green fluorescence signals. It was also worth mention that sgRNA linked *pX330* plasmids were cloned and the confirmation of the knockout of target *lep* gene sequences in HEK293 cells can be performed.

To disrupt the mouse lep gene, pCAG-EGxxFP-target containing the 420 bp exon 3 sequence of the *lep* gene was transfected with pX330-sgRNA vector. The fluorescence of EGFP was observed to determine the particular sgRNA sequence which induces the DSB of the pCAG-EGxxFP-target vector. The pX330-sgRNA vectors with scores 3, 4 were transferred to HEK293 cells to select the two most effective sgRNA-pX330 plasmids. As a result, two candidate sequences (sgRNA 28, sgRNA 95) were most effective in generating a suitable DSB. Therefore, we screened this pair of sgRNAs that target exon 3 to produce homozygous *lep* knockout embryos. To decrease off-

target sites while increase on-target potential, we thoroughly investigated exon 3 using sgRNA designing software. Simultaneous usage of two independent sgRNAs induces a 167 bp loss mutation on the lep exon 3 gene, inducing a frameshift mutation and disrupting the LEP protein structure. When comparing with the 167 amino acid wild-type protein, the 54 amino acids were eliminated in the mutant peptide, indicating that the mutated leptin protein is not working properly.

Here, we successfully designed 2 sgRNAs that have the most efficient and the least off-target potentials. This sgRNA pair will be used to generate the *lep* KO mouse model by microinjecting the CRISPR-Cas9 system into 2 pronuclear embryos, efficiently create adult mice carrying a mutation of the *lep* gene, allowing for the rapid knockout-animal production.

Using genomic engineering to modify a fertilized zygote is a faster and more effective approach to generate chimeric animal models than the conventional modified embryonic stem cells method (Cohen, 2016). Because the traditional gene editing strategies need eight months to generate the chimeric animal (Fig. 5) (Cohen, 2016). This technique accelerates the production of KO lep mice by breeding rapidly as well as reduced price, decreased difficultly technical steps, and more efficiency for the generation of mutant mouse models. Therefore, the genetic obesity-diabetes mice production using the CRISPR/Cas9 system helps overcome some disadvantages of traditional breeding methods (Pelletier, Gingras *et al.*, 2015).

Conclusion

468 candidate oligos were screened and checked in silico for the properties to be sgRNAs for CRISPR/Cas9 procedure to knock out mouse's lep gene. Of these, four sgRNAs (sgRNA 7, -28, -66, -95) were chosen for further validation *in vitro*. The sgRNA28 and sgRNA95 showed the most specific shear potential in vitro model, inducing the removal of 167 bp fragment on the exon 3 of lep gene. This pair of sgRNA was promising and would be validated further for the generation of genetic obesity-diabetes mice models.

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