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Generation of CRISPR-cas9 construct for knockout of genes encoding chromatin-associated proteins

Mumtaz Aarif Wani[1] and Aijaz Ahmad Ganaie[2*]

Abstract

Background: Eukaryotic genomes have a multiscale three-dimensional organization varying from nucleosomes, loops, topologically associating domains, and chromosome territories. Chromatin, DNA wrapped around histone proteins, helps in packaging long DNA within tiny nuclear spaces. We used CRISPR-cas9, which is a system of single-protein and single-guide RNAs for genome engineering and also is simple and target specific.

Method: Two major protein families involved in maintaining and regulating structure and dynamics of chromatin are trithorax group (TrxG) and polycomb group (PcG) proteins. This study was undertaken to generate knockout cell lines of some TrxG and PcG proteins using the CRISPR-based approach in order to study their role in higher order chromatin organization.

Results: From TrxG, ISWI and Acf were selected, and from PcG, Pc and Psc were selected. Three pAc-sgRNA-Cas9-puro-vector constructs for ISWI gene, one pAc-sgRNA-cas9-puro-vector construct for Pc, gene and two pAc-sgRNA-cas9-puro-vector constructs for each of the Acf and Psc gene were generated. These constructs were confirmed by PCR and sequencing.

Conclusion: In the future, these constructs will be used to study the role of their respective target genes in chromatin organization.

Keywords: Trithorax group proteins, Polycomb group proteins, CRISPR-cas9

Introduction

Chromatin is a complex structure consisting of DNA, proteins, and RNA. Chromatin is packaged into small nuclear space in the cell, but at the same time chromatin is accessible to various transcription factors. This dynamic nature of chromatin is maintained by various proteins and protein complexes. The structural proteins, called histones, help in the DNA assembly. The core histones, namely H2A, H2B, H3, and H4, form the nucleosome, which is the basic unit of chromatin organization. The nucleosome consists of an octamer of core histones made of a pair of each core histone and a DNA fragment of 146 bp wrapped around the octamer core. This forms the "beads on a string" structure of chromatin, then nucleosomal chromatin to a compact structure called 30-nm fiber chromatin (heterochromatin). The 30-nm fiber is packaged to a higher level into the metaphase chromosome. Chromatin-associated proteins are of two counteracting groups, trithorax group (TrxG) and polycomb group (PcG) that maintain the cellular memory. TrxG is of critical importance in the senescence, DNA damage, epigenetic regulation of the cell cycle, and stem cell biology (Ingham 1998). Trithorax chromatin regulators are evolutionarily conserved proteins and can be divided into three classes on the basis of their molecular function. One class, methylate histone tails, contains a SET domain. The second class, ATP-dependent chromatin remodeling factors, includes proteins that can read the histone methylation marks that are laid down by the SET domain proteins. The third class can directly bind to specific DNA sequences and includes TrxG proteins comprised of

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Fig. 1 Picture showing position of guide sequences for the selected sequence of a gene

some histone modifiers and chromatin remodelers, as well as proteins that are not categorized within the first two classes. Thus, most of the Trx proteins perform their functions as a part of their large multiprotein complexes that are histone modifiers or nucleosome remodelers, to regulate gene expression. PcG implement transcription silencing in higher eukaryotes by forming chromatin-modifying complexes. Two main families of complexes, called polycomb repressive complex (PRC) 1 and PRC2, are targeted to repress DNA regions. We used CRISPR-cas9 technology to generate pAc-sgRNAcas9-puro-vector knockout constructs for some proteins of these two groups (TrxG and PcG) to study their function. Clustered regularly interspaced short palindromic repeats [CRISPR] are DNA sequences found in prokaryotic genomes (Grissa et al. 2007). Each repeat is followed by a "spacer DNA," which is adopted from a previous infection by a phage or plasmid (Marraffini and Sontheimer 2010). Simply, CRISPR-Cas9 is an immune system in prokaryotes and provides a form of acquired immunity against phages and plasmids. CRISPR-associated genes [Cas genes] are a set of genes that are closely associated with CRISPR. Bacteria which are able to survive a viral infection fragmentize the viral DNA and then integrate few of these fragments into their CRISPR loci and also pass on this genetic information generation to generation. Whenever in the future the same bacteria or its progeny is infected by the same viral strain or another viral strain which shares the same genetic information which the bacteria has integrated into its CRISPR locus are able to survive the infection with the help of CRISPR activation, the RNAs transcribed from CRISPR loci called CRISPR RNA [crRNA] (Pennisi 2013) or guide RNA. RNA makes a stable complex with Cas protein and navigates and binds the specific target in viral DNA and makes a double-strand break, hence degrading the viral DNA and, in this way, escaping viral invasion. *Streptococcus pyogenes* has a CRISPR system and a single-cas gene called cas9 that is required for the function of this CRISPR system in the bacterium. Cas9 is a fascinating enzyme. Cas9 has the ability to interact with DNA and generate a double-strand break [DSB] at sequences that match the guide RNA sequences. The guide RNA base pairs with protospacer in viral DNA to allow cas9 enzyme to cause a specific double-strand break. The guide RNA also base pairs with another RNA sequence called tracrRNA to form a structure that recruits the cas9 enzyme. Therefore, two RNAs, guide RNA and tracrRNA, and cas9 enzyme are required to form a complex that recognizes the viral DNA and degrades it. In 2012, Jinek et.al linked together guide RNA and tracrRNA molecules to form a single-guide RNA [sgRNA], to generate a system of single-protein and single-guide RNA for genome engineering. This new genome engineering technology is simply called CRISPR-cas9 technology. This technique is used to generate knockout cell lines which in turn provide the information regarding the function of the same gene (Jinek et al. 2012).

CRISPR/Cas9 systems have been successfully used for precise genome editing in mouse embryonic stem cells (ESCs) and targeted biallelic mutagenesis in F0 zebrafish (Jao et al. 2013; Wang et al. 2013; Yang et al. 2013). Recently, CRISPR-Cas9 systems have been used to efficiently edit the genome of adult mice in vivo in the liver and muscle (Long et al. 2014; Xue et al. 2014; Yin et al. 2014). The RNA-guided Cas9 endonuclease induces sequence-specific DNA double-strand breaks (DSBs) that are repaired mostly by the classical and alternative nonhomologous end-joining pathways (c-NHEJ or AEJ) or by homology-directed repair (HDR) (Boboila et al. 2012). This study was carried out to generate the knockout cell lines of chromatin-associated proteins, viz, polycomb and trithorax. The constructs for some of these genes were successfully generated which will be used in the future to generate the knockout cell lines of the same. This study will provide insight into the mechanism how these proteins are responsible for the chromatin structure and function.

This study was carried out to design and synthesize guide sequences for TrxG (ISWI and Acf) and PcG (Pc and Psc) proteins and to generate CRISPR-cas9 constructs to knockout TrxG (ISWI and Acf) and PcG (Pc and Psc) genes.

	score	sequence	
Guide #1	95	ATACTGAAAGTATAACTAAC	AGG
Guide #2	94	GATTTTTTTTAATGAATGGT	GGG
Guide #3	92	CGATTTTTTTTAATGAATGG	TGG
Guide #4	85	ATGCGATTTTTTTTAATGAA	TGG
Guide #5	81	ACTTTCAGTATATAAATAAA	AGG

Fig. 2 Guide sequences with their scores

Table 1 Guide sequences of genes under study

S. no.	Name of the target gene	No. of guides	Name of guides	Guide sequence 5′ to 3′
01.	ISWI	03	ISWI19, ISWI20, ISWI21, ISWI22 and ISWI23	TTCGACGGCAGCTGTATCTGTT, TTCGGGTCTACCCTTAGGCTTCGT, TTCGATATCTCCGTCTGCTTTAGC, TTCGCTTTTCACCGGATGAACTGG, TTCGTAAGAGTCCCACGAAGCCTA
02.	Acf	02	Acf10 and Acf11	TTCGCCTTCGGGCACCCGTCCTTC, TTCG GGTGTTGACCGCCGACTGTT
03.	Pc	02	Pc1, Pc2	TTCGGCCGCGTACACTAGATCGAC, TTCG TCCAGTCGATCTAGTGTACG
04.	Psc	02	Psc8 and Psc9	TTCGGGCTGTATTGCTTTCGATTC, TTCG GACAACGGCAACGAATAAGG

Methodology

Designing guide oligos

Select 23-250NT sequence of your desired gene and paste it in a web tool called crispr.mit.edu. Download the guide sequences and choose the guides on the basis of score of guides (Figs. 1 and 2). If target sequence does not begin with G, add G to its 5′ end to allow transcription from the U6 promoter. For the forward oligonucleotide, add TTC to the 5′ end of the target sequence. For the reverse oligonucleotide, reverse complement the target sequence (including an additional G nucleotide), and add AAC to the 5′ end. Synthesize the oligonucleotides. Oligos were synthesized as shown in Table 1.

Annealing of oligos

The forward and reverse oligos were annealed using PCR machine as follows: Mix 10 μl of 2x annealing buffer (20 mM Tris, 2 mM EDTA, 100 mM NaCl, pH 8.0) with 5 μl each of 100 uM forward and reverse oligos.

Annealing program

The oligos are annealed as shown in Table 2. Incubate for 30 min at 37 °C. Dilute 10x in ddH_2O.

Phosphorylation of annealed oligos

After annealing, the oligos were phosphorylated using annealed oligos (1 μl, 10 μM), Roche T4 DNA ligase buffer (1 μl, 1x, contains ATP), T4 polynucleotide kinase (1 μl, 1 U), ddH_2O (7 μl) to make the final volume 10 μl and incubate at 37 °C for 10 min.

Table 2 Annealing program

98 °C	1 min
98–88 °C	5 s, decrease 0.1 °C/cycle × 99 cycles
88–78 °C	10 s, decrease 0.1 °C/cycle × 99 cycles+
78–68 °C	10s, decrease 0.1 °C/cycle × 99 cycles
68–58 °C	10s, decrease 0.1 °C/cycle × 99 cycles
58–48 °C	10 s, decrease 0.1 °C/cycle × 99 cycles
48–38 °C	10 s, decrease 0.1 °C/cycle × 99 cycles
38–18 °C	10 s, decrease 0.1 °C/cycle × 99 cycles
18 °C	Forever

Digestion of pAc-sgRNA-cas9-puro-vector

Meanwhile, 2 μg pAc-sgRNA-Cas9 vector (300 ng, 12 μl) was digested for 1 h at 37 °C using NEB buffer 4 (1x, 5 μl), BspQ1 (NEB, 2 μl), and ddH_2O (31 μl) to make the final volume 50 μl. Run the digested vector on agarose gel and purify the digested vector using a kit by the manufacturer's protocol.

Ligation of digested vector pAc-sgRNA-Cas9-puro and phosphorylated oligos

The ligation was performed using pAc-sgRNA-Cas9-puro-vector (2 μl), 10x diluted oligos (2 μl), Roche T4 DNA ligase buffer (1 μl), T4 DNA ligase (1 μl), and ddH_2O (4 μl) to make the final volume 10 μl and incubate at 18 °C for 2 h.

Transformation

Transform 2 μl of ligated vector (with desired oligos) to a 50 μl aliquot of chemically competent DH5-α cells by:

- Incubate 20 min on ice with plasmid.
- Heat shock for 30 s at 42 °C.
- Cool for 2 min on ice.
- Use LB-ampicillin/carbenicillin plates (100 μg/ml).
- Incubate at 37 °C overnight.

Table 3 CRISPR-CAS9 constructs to knockout TrxG and PcG genes+

S. no	Name of the target gene	No. of guides	Name of guides	Conformation by PCR	Conformation by sequencing
1	ISWI	03	ISWI21,	YES	YES
			22,	YES	YES
			23	YES	YES
2	Acf	02	Acf 10	YES	YES
			Acf11	YES	YES
3	Pc	02	Pc2	YES	YES
4	Psc	02	Psc8	YES	YES
			Psc9	YES	YES

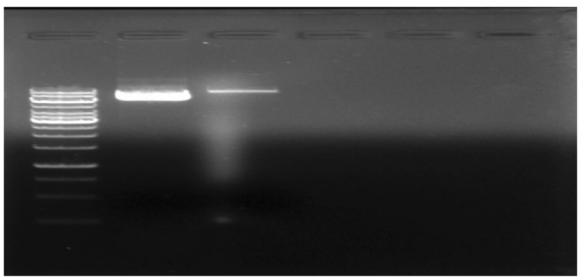


Fig. 3 1% agarose gel of undigested and digested pAc vector

- The next day, three colonies for each clone were randomly selected and inoculated in 5 ml LB broth containing ampicillin 1 μg/ml. The tubes were kept in a shaking incubator at 37 °C overnight.
- Isolation of plasmids was done using Qiagenmini prep kit.
- The integrity of the plasmids was checked on agarose gel.

Confirmation of positive constructs by PCR and sequencing

Confirmation of positive constructs was done by PCR using buffer (1x, 5 μl), dNTPs (1.5 mM, 5 μl), Forward primer (U6F, 0.25 μM, 1.25 μl), Reverse primer (0.25 μM, 1.2 μl), Template (50 ng, 1 μl), Taq. Pol. (0.05, 0.1 μl), and MQ (36.4 μl) to make the final volume 50 μl. The PCR program followed was hot start (95 °C, 5 min), denaturation (95 °C, 30 s), annealing (56 °C, 30 s), extension (72 °C, 25 s), and last extension (72 °C, 7 min).

The positive pAc-sgRNA-Cas9-puro-vector constructs were sent to Scigenom (Kochi, India) for sequencing. The sequencing was checked by alignment using Emboss needle web tool.

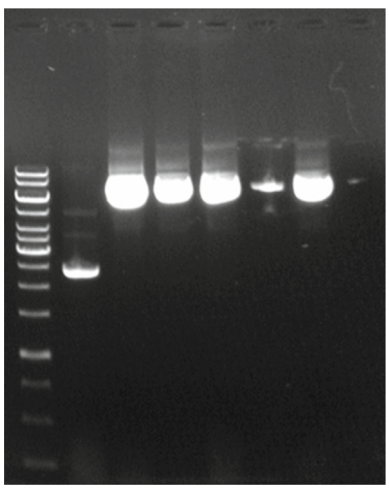
Results and discussion

Eight CRISPR-CAS9 constructs to knockout TrxG and PcG genes were successfully generated as shown in Table 3.

Generation of pAc-sgRNA-Cas9-puro-vector TrxG (ISWI and Acf) constructs

In order to generate CRISPR-based knockout cell lines of ISWI, the pAc vector was digested with BspQ1, as shown in Fig. 3. The digested vector and the annealed, phosphorylated oligos were ligated. We first ligated five phosphorylated guides of ISWI, i.e., ISWI19, ISWI20, ISWI21, ISWI22, and ISWI23, with the digested vector. Next, transformation of the same was performed in DH5-alpha competent cells. After transformation, DH5-alpha cells were grown on LB-ampicillin agar plates. We got a good number of colonies only for ISWI19, 21, 22, and 23. Three colonies from each plate were randomly selected and inoculated in 5 ml LB-ampicillin broth. The tubes were kept at 37 °C in a shaking incubator overnight. The next day, recombinant plasmids were isolated using miniprep kit. On running the isolated recombinant plasmids on 1% agarose gel, only ISWI21, 22, and 23 showed positive clones as shown in Fig. 4. The pAc-sgRNA-cas9-puro-vector was ran as the control. The confirmation of positive clones was further confirmed by PCR and sequencing sequentially. For the PCR confirmation, the U6F and the complementary oligo of guide sequences were used as primers and the PCR products were run on 1% agarose gel, as shown in Fig. 5. For the confirmation by sequencing U6F primer was used, the sequencing results are shown in Fig. 6a–c.

For the generation of knockouts of TrxG gene Acf, two different guide sequences were selected for Acf. These two guide sequences were annealed with their complementary guide sequences and phosphorylated. Meanwhile, the pAc-sgRNA-Cas9-puro-vector was



Lane L: 1 kb ladder; Lanes: 1, 2, 3, and 4: ISWI19, ISWI21, ISWI22 and ISWI 23 respt. Lanes: 5 and 6: pAc-sgRNA-cas9-puro-vector

Fig. 4 1% agarose gel of recombinant plasmids of ISWI guide sequences

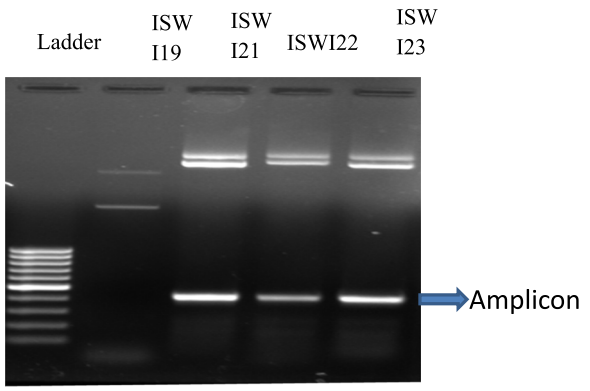


Fig. 5 1% agarose gel showing PCR confirmation of positive pAc-sgRNA-Cas9-puro-vector knockout

designed with BspQ1 restriction enzyme as mentioned in the materials and methods, purified and ligated with the guide oligos. Transformation of the ligated plasmids was performed in DH5-alpha competent cells and plated on LB-ampcillin agar plates. The next day, single colonies, two from each, were inoculated in LB broth and were grown overnight at 37 °C in a shaking incubator. Recombinant plasmids as shown in Fig. 7a, were isolated and the integrity of plasmids was checked on 1% agarose gel. As evident from Fig. 7a, the impurities were found in the plasmids and the same experiment was repeated as shown in Fig. 7b. The Acf10 and Acf11 constructs were confirmed for the presence of insert by PCR and sequencing. The PCR was performed using U6F primer and the complementary sequence of each oligo. The PCR

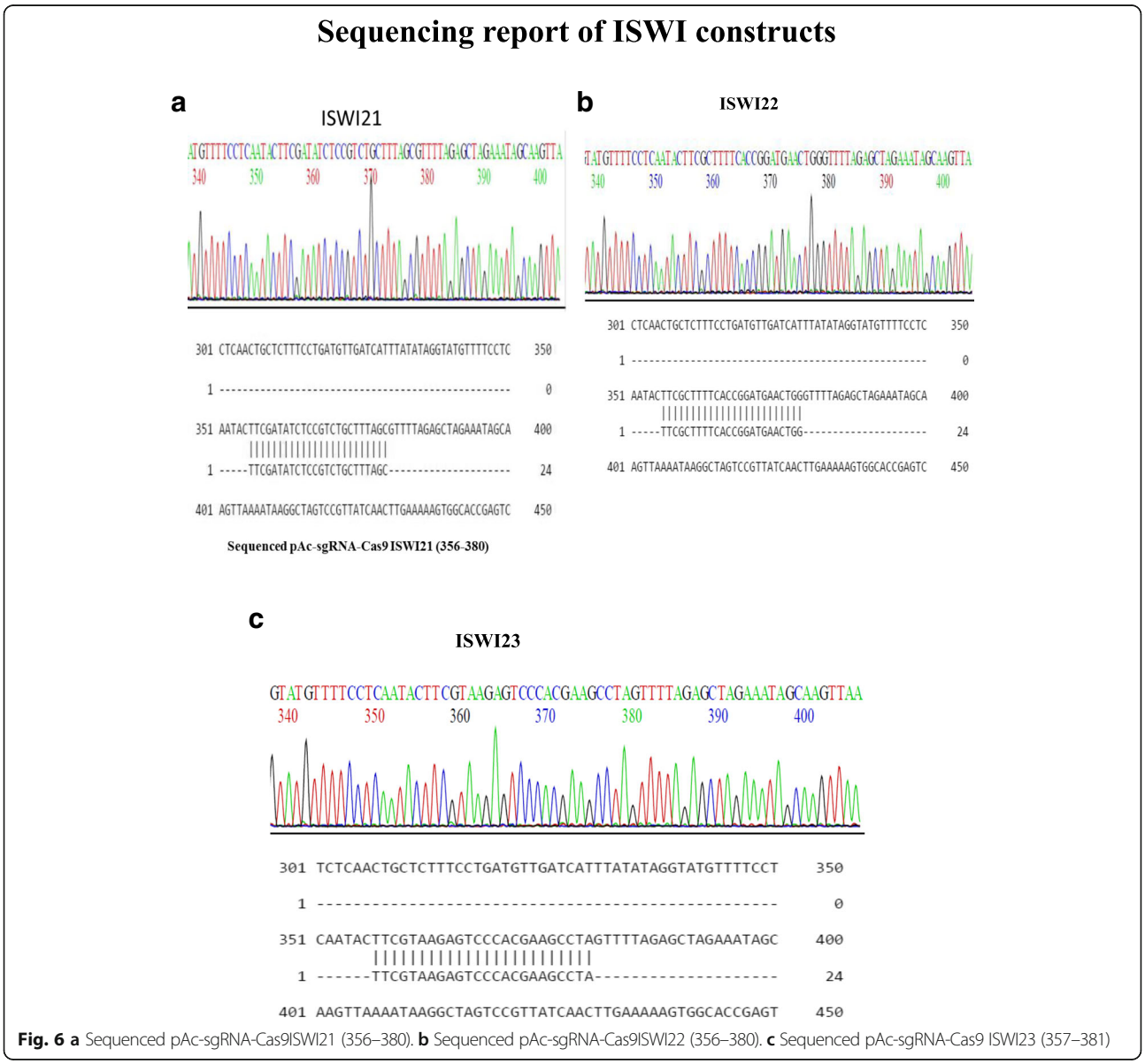


Fig. 6 **a** Sequenced pAc-sgRNA-Cas9ISWI21 (356–380). **b** Sequenced pAc-sgRNA-Cas9ISWI22 (356–380). **c** Sequenced pAc-sgRNA-Cas9 ISWI23 (357–381)

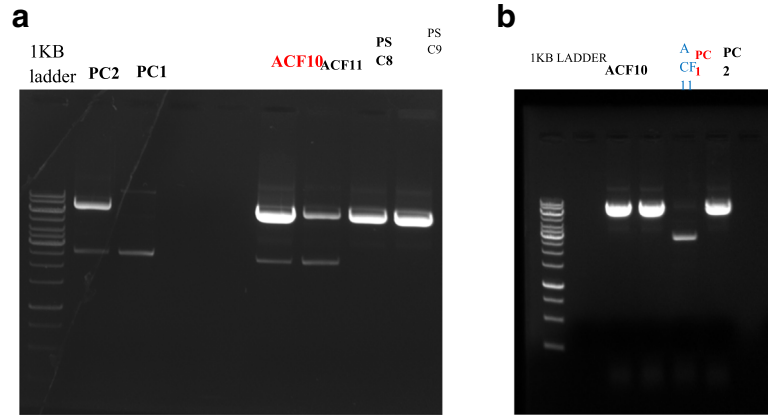


Fig. 7 **a** and **b** 1% agarose gel of positive pAc-sgRNA-Cas9-puro-vector constructs for Pc, Acf and Psc genes

products were run on 1% agarose gel keeping pAc-sgRNA-cas9-puro-vector as the negative control as shown in Fig. 8a. The size of the PCR fragment was found almost 491 bp as expected in each case. Further confirmation of the recombinants was performed by sequencing using U6F primer as shown in Fig. 9b, c.

Generation of CRISPR-Cas9 constructs for polycomb group [PcG] proteins [Pc and Psc]

For the generation of knockouts of PcG genes, Pc and Psc, and one and two different guide sequences, respectively, were selected for each gene. These guide sequences were annealed with their complementary guide sequences and phosphorylated as mentioned in the materials and methods. Meanwhile, the pAc-sgRNA-Cas9-puro-vector was digested with BspQ1, purified and ligated with the guide oligos. Transformation of the ligated plasmids was performed in DH5-alpha competent cells and plated on LB-ampcillin agar plates. The next day, single colonies, two from each, were inoculated in LB broth and were grown overnight at 37 °C in a shaking incubator. Recombinant plasmids as shown in Fig. 7a were isolated, and the integrity of plasmids was checked on 1% agarose. As evident from Fig. 7a, the impurities were found in the plasmids except Psc8 and Psc9.The same experiment was repeated for the generation of Pc1 and Pc2 constructs. The recombinant plasmids of these genes were run on 1% agarose gel, as shown in Fig. 7b. The impurity was still found in the case of Pc1. The Pc2, Psc8, and Psc9 constructs were confirmed for the presence of insert sequentially by PCR and sequencing. The PCR was performed using U6F primer and the complementary sequence of each oligo. The PCR products were ran on 1% agarose gel keeping pAc-sgRNA-cas9-puro-vector as the negative control as shown in Fig. 8a, b. The size of the PCR fragment was found almost 491 bp as expected in each case. Further confirmation of the recombinants was performed by sequencing using U6F primer as shown in Fig. 9a, d, e.

Discussion

The CRISPR/Cas9 system is proven to be a powerful yet simple tool to manipulate the genome for the generation of genetically modified animals. In this study, we report the successful generation of eight CRISPR-cas9 constructs to knockout TrxG and PcG. We synthesized five guide sequences ISWI19, ISWI20, ISWI21, ISWI22, and ISWI23 for TrxG protein, ISWI. Among the five guides sequences, only three ISWI21, ISWI22, and ISWI23 successfully integrated into pAc-sgRNA-cas9-puro-vector to form three CRISPR-cas9 constructs for ISWI gene.

Chromatin-associated proteins are of two counteracting groups, trithorax group (TrxG) and polycomb group (PcG), that maintain the cellular memory. TrxG is of critical importance in the senescence, DNA damage, epigenetic regulation of the cell cycle, and stem cell biology. Trithorax chromatin regulators are evolutionarily conserved proteins and can be divided into three classes on the basis of their molecular function. One class is methylate histone tails, and it contains SET domain. The second class, ATP-dependent chromatin remodeling factors, includes proteins that can read the histone methylation marks that are laid down by the SET domain proteins. The third class can directly bind to specific DNA sequences and includes TrxG proteins comprised of some histone modifiers and chromatin remodelers, as well as proteins that are not categorized within the first two classes. Thus, most of the Trx proteins perform their functions as a part of their large multiprotein complexes that are histone modifiers or nucleosome remodelers, to regulate gene expression. PcG implement transcription silencing in higher eukaryotes by forming chromatin-modifying complexes. Two main families of complexes, called polycomb repressive complex (PRC) 1 and PRC2, are targeted to repress DNA

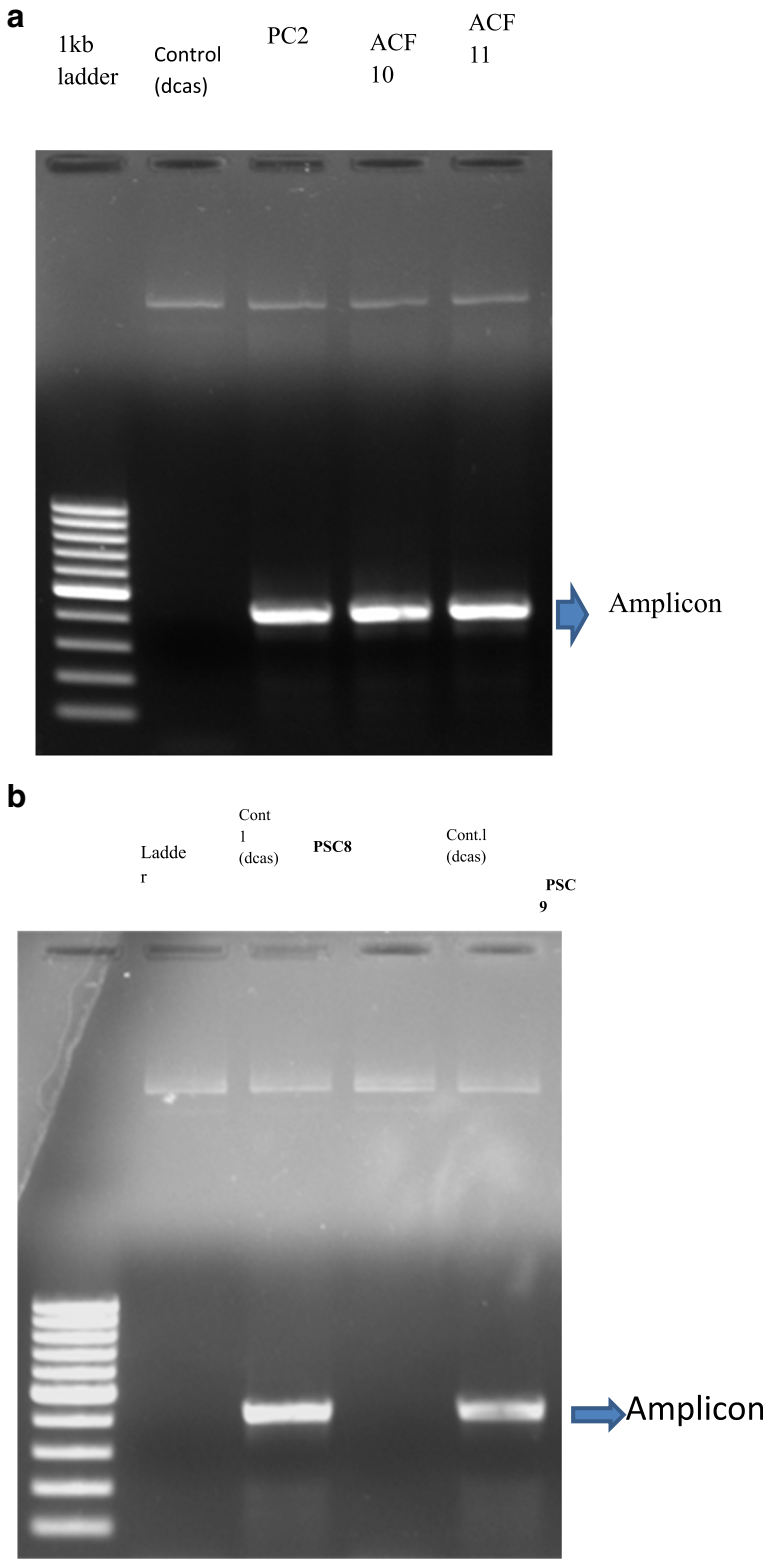


Fig. 8 **a** 1% agarose gel showing PCR confirmation of pAc-sgRNA-Cas9-puro-vector–positive construct for PC and ACF genes. **b** 1% agarose gel showing PCR confirmation of positive pAc-sgRNA-Cas9-puro-vector construct for PSC gene

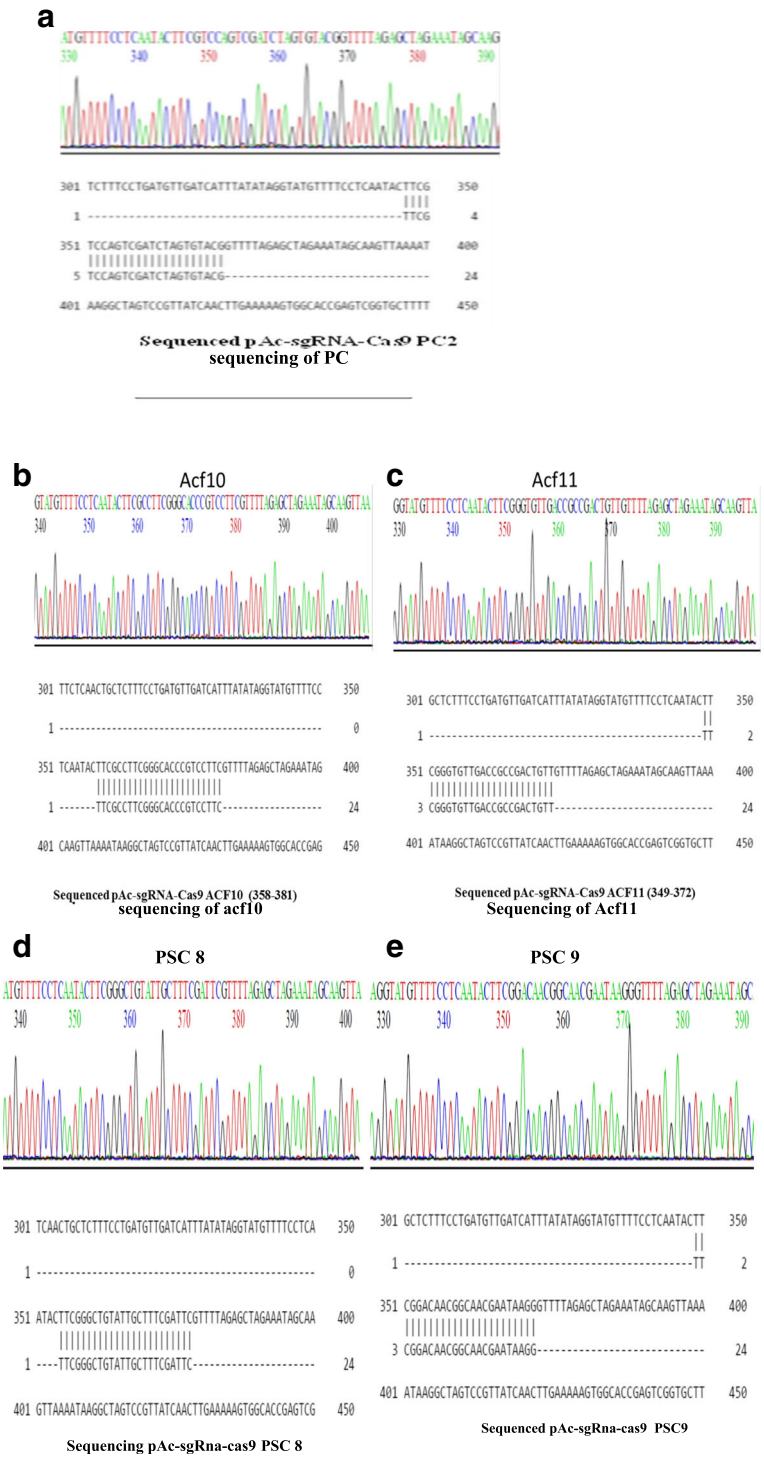


Fig. 9 **a** Sequencing of PC. **b** sequencing of acf10. **c** Sequencing of Acf11. **d** Sequencing pAc-sgRna-cas9 PSC 8. **e** Sequenced pAc-sgRna-cas9 PSC9

regions. We used CRISPR-cas9 technology to generate pAc-sgRNAcas9-puro-vector knockout constructs for some of the proteins of these two groups (TrxG and PcG) to study their function. In 2012, Martin Jinek et al. linked together guide RNA and tracrRNA molecules to form a single-guide RNA [sgRNA] to generate a system of single-protein and single-guide RNA for genome engineering. This new genome engineering technology is

simply called CRISPR-cas9 technology. This technique is used to generate knockout cell lines which in turn provide the information regarding the function of the same gene.

The first response of CRISPR-Cas system is to capture viral DNA and select spacers from it to insert them into CRISPR locus (Tyson and Banfield 2008). Cas1 and Cas2 are found in all three CRISPR-Cas immune systems that indicate they are involved in spacer adaptation. This hypothesis was confirmed by mutation studies (Aliyari and Ding 2009; Dugar et al. 2013; Hatoum-Aslan et al. 2011). There are multiple Cas1 proteins with diverse amino acid sequences, but similar crystal structures. All purified Cas1 proteins are metal-dependent nucleases/integrases and show sequence independent binding to DNA (Wiedenheft et al. 2012).After analysis of repeat-spacer array, it was found that the spacers which were selected from phage genomes were not randomly selected, but only those regions called protospacers are selected for adaptation in the CRISPR locus. These protospacers lie upstream adjacent to short[3-5 bp] DNA sequences called protospacer-adjacent motifs[PAMs]. Analysis of CRISPR-Cas systems showed that PAMs are important for type I and type II systems only (Horvath et al. 2008; Deveau et al. 2008). Different CRISPR-Cas systems show conservation of the PAM sequences they recognize. New spacers are preferentially added adjacent to leader sequence in a directional manner (Shah et al. 2009). A single long primary transcript is transcribed from the promoter in the leader sequence. CRISPR RNAs [crRNAs] are formed from this single transcript by cleavage inside the repeat sequence. The mechanism of production of crRNAs is different in the three CRISPR systems.

Conclusion

We synthesized five guide sequences ISWI19, ISWI20, ISWI21, ISWI22, and ISWI23 for TrxG protein, ISWI. Among the five guides sequences only three ISWI21, ISWI22, and ISWI23 successfully integrated into pAc-sgRNA-cas9-puro-vector to form three CRISPR-cas9 constructs for ISWI gene. Two guide sequences, Acf10 and Acf11, were synthesized for another TrxG protein; Acf and both the guide sequences successfully integrated into the pAc-sgRNA-cas9-puro-vector to form two CRISPR-cas9 constructs for Acf gene. For PcG proteins, Pc and Psc, two guide sequences for each Pc1 and Pc2, and Psc8 and Psc9 were synthesized, respectively. Among Pc1 and Pc2, only Pc2 successfully integrated into pAc-sgRNA-cas9-puro-vector to form single-CRISPR-cas9 constructs for Pc gene. Both the guide sequences synthesized for Psc gene successfully integrated into pAc-sgRNA-cas9-puro-vector to form two CRISPR-cas9 constructs for Psc gene. Finally, in total, we successfully generated eight CRISPR-cas9 constructs, five for TrxG proteins, and three for PcG proteins (Table 1).

Abbreviations

CRISPR: Clustered regulatory interspersed short palindromic repeats; DSB: Double-strand break; ISWI: Imitation switch; PcG: Polycomb group; PRC: Polycomb-repressive complex; RNA: Ribonucleic acid; TrxG: Trithorax group

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Availability of data and materials

The necessary data was collected during study. However, on request or proposal, additional information will be provided by corresponding author.

Authors' contributions

Experimental work was carried out by MAW, while as with data compilation, editing was done by AAG. Both authors read and approved the final manuscript.

Ethics approval and consent to participate

NA.

Consent for publication

NA.

Competing interests

The authors declare that they have no competing interests.

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