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Temperature effect on CRISPR-Cas9 mediated genome editing

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ABSTRACT

Zinc-finger nuclease (ZFN), transcription activator-like effector nuclease (TALEN), and clustered, regularly interspaced, short palindromic repeats/ CRISPR-associated protein 9 (CRISPR-Cas9), are the most commonly used genome editing tools. Previous studies demonstrated that hypothermia treatment increased the mutation rates induced by ZFNs and TALENs in mammalian cells. Here, we characterized the effect of different culture temperatures on CRISPR-Cas9 mediated genome editing and found that the genome editing efficiency of CRISPR-Cas9 is significantly hampered by hypothermia treatment, unlike ZFN and TALEN. In addition, hyperthermia culture condition enhances genome editing by CRISPR-Cas9 in some cell lines, due to the higher enzyme activity and sgRNA expression level at higher temperature. Our study has implications on CRISPR-Cas9 applications in a broad spectrum of species, many of which do not live at 37°C.

KEYWORDS: CRISPR-Cas9, Hyperthermia, Genome editing, Mammalian cells

INTRODUCTION

Zinc-finger nuclease (ZFN) (Urnov et al., 2010), transcription activator-like effector nuclease (TALEN) (Bogdanove and Voytas, 2011), and clustered, regularly interspaced, short palindromic repeats/CRISPR-associated protein 9 (CRISPR-Cas9) (Doudna and Charpentier, 2014; Hsu et al., 2014) are invaluable tools for genome editing. When introduced into cells, these nucleases can induce DNA double-strand breaks (DSB) at specified genomic loci. DSBs are repaired either by non-homologous end joining (NHEJ), or by homology-directed repair (HDR), leading to various types of genetic modifications (Wiles et al., 2015). Many efforts have been made to improve the performance of these nucleases. One simple but robust method is to culture the cells in transient hypothermia conditions (30°C), which can significantly enhance ZFN and TALEN-mediated genome editing efficiency (Doyon et al., 2010; Carlson et al., 2012). However, how temperature affects the efficacy of the CRISPR-Cas9 system has not been characterized.

Here, we characterize the effect of temperature on CRISPR-Cas9 mediated genome editing and show that the genome editing efficiency of CRISPR-Cas9 is substantially hampered by hypothermia treatment, while hyperthermia (39°C) culture can enhance the efficiency in some cell lines. We also analyze the underlying mechanisms of this phenomenon.

RESULTS

Comparing ZFN, TALEN and CRISPR-Cas9 genome editing efficiency at the AAVS1 locus at different temperatures

First, we compared the genome editing efficiency of ZFN, TALEN and CRISPR-Cas9 systems at the *AAVS1* (adeno-associated virus integration site 1) locus at different culture temperatures. Based on the previously published ZFN and TALEN target site (Hockemeyer et al., 2009, 2011), we designed the CRISPR single guide RNA (sgRNA) so that all three types of nucleases would generate DNA DSBs at very similar sites at the *AAVS1* locus (Fig. 1A and Table S1). K562 cells were grown to the optimal density at 37°C, and then electroporated with plasmids encoding each nuclease system along with pMAX-GFP plasmid (Lonza, Germany) using Lonza 4D-nucleofector X. After nucleofection, cells were equally divided into four parts, and cultured at 30°C, 33°C, 37°C, and 39°C independently. Three days later, samples were collected for FACS analysis. The transfection efficiency was consistent at different temperatures, while higher temperature led to higher cell number (Fig. S1).

Next, we utilized the Surveyor assay (Guschin et al., 2010) to analyze genome editing efficiency. As previously reported (Doyon et al., 2010; Carlson et al., 2012), ZFN treated samples cultured at 30 °C and 33°C had higher genome editing efficiency than samples cultured at 37°C (Fig. 1B). The temperature effect in TALEN treated samples was similar but not as evident (Fig. 1C). Surprisingly, we found that the CRISPR-Cas9 system performed in an opposite manner, with higher culture temperature leading to a higher editing efficiency (Fig. 1D). Gene editing efficiency improved from 11.5% at 30°C to 18.7% at 37°C, suggesting a lower temperature impeded the CRISPR-Cas9 activity. Moreover, 39°C culture conditions resulted in editing efficiency higher than 37°C culture (Fig. 1D).

Evaluating multiple target sites in K562 cells

To verify whether the same effects can be observed at other genomic loci, we tested nine different sgRNAs targeting different genes in K562 cells (Table S1). The overall effect of culture temperature on genome editing efficiency is similar to the *AAVS1* results (Figs. 1E and S2). Notably, several sgRNAs leading to robust gene editing at 37°C were barely functional at 30°C. Meanwhile, 39°C culture led to significantly higher indel (insertions or deletions) frequency compared to 37°C culture at several loci (Figs. 1E and S2).

To test whether the plasmid dosage contributes to the temperature effect on genome editing efficiency, we tested two dosages of plasmids (2 µg and 5 µg) encoding either TALEN or CRISPR-Cas9 systems, targeting *OCT4* and *MECP2* loci, and cultured the cells at different temperatures. For both systems at each temperature, the overall editing efficiency increased with increasing plasmid amount. The temperature effect was similar using either dosage, with higher culture temperature leading to more efficient CRISPR-Cas9 activity and less robust TALEN mediated gene editing (Fig. S3).

Evaluating multiple cell lines

To verify our findings in other cell types, we targeted multiple genes in several commonly used cell lines including human embryonic stem cells (ESCs). In accordance with the results acquired from K562 cells, we found that CRISPR-Cas9 genome editing efficiencies increased at higher temperature in Jurkat cells. The indel frequencies at the *AAVS1* and *B2M* loci at 39°C were significantly higher than those at 37°C culture (Fig. 2A). While we did not observe the efficiency improvement of the 39 °C

culture in the other cell lines we tested, we observed a consistent editing efficiency improvement from 30°C to 37°C (Figs. 2B and S4).

Temperature effect on TALEN and CRISPR mediated HDR

Next we determined the effect of culture temperatures on HDR efficiency using *OCT4* and *MECP2* as targets. The HDR templates used in these experiments were single-stranded oligo nucleotide DNA (ssODN), inserting one nucleotide to form a restriction site flanked by 42 nt homology sequences (Figs. 3A and S5A). First, we determined the total indel frequencies of samples with and without donor oligo under different temperatures by TIDE (Tracking Indels by DEcomposition) analysis (Brinkman et al., 2014), and the trends were similar to previous results (Fig. S6).

Then we quantified the HDR efficiency using restriction fragment length polymorphism (RFLP) and TIDE analysis. Using both methods, we found that CRISPR-Cas9 mediated HDR efficiency increased along with the temperature increasing from 30°C to 39°C (Fig. 3B). The HDR efficiency of TALEN also increased from 30°C to 37°C, while decreased at 39 °C (Fig. 3C). For both CRISPR-Cas9 and TALEN, the same temperature effect on HDR efficiency was observed at the *MECP2* locus (Fig. S5B).

Temperature effect on off-target frequency of CRISPR-Cas9

One major concern of genome editing is the frequency of off-target edits. To evaluate the impact of hyperthermia treatment on off-target activity, we investigated the *EMX1* and *VEGFA* gene to check whether higher culture temperature would increase off-target editing as well. For each gene, we tested three off-target sites with high off-target frequencies described in previous studies (Cho et al., 2014; Hsu et al., 2013). In two out of three *EMX1* off-target sites and one out of three *VEGFA* off-target sites, we observed detectable off-target activity in K562 cells. Consistent with on-target efficiency, off-target efficiency in all three sites also showed higher indel frequency at 39°C than 37°C culture (Fig. S7). These results suggest that the overall activity of the CRISPR-Cas9 system is higher at higher culture temperature.

The mechanism of temperature effect on CRISPR-Cas9 mediated genome editing

To understand the mechanism of temperature effect, we first tested the activity of Cas9-guide

RNA ribonucleoprotein (RNP) under different temperatures *in vitro*, via digestion of linearized plasmids containing sgRNA target sequence. We incubated samples for either 60 min or 10 min, and in both cases found that a lower temperature led to lower Cas9 nuclease activity (Fig. 4A), indicating that lower temperature has a negative but rather weak effect on RNP mediated DNA cleavage. When we electroporated Cas9 RNP (Wang et al. 2016) into K562 cells and cultured them at different temperatures, the indel frequency at 39°C or 37°C was higher than that at 30 °C or 33 °C, but the effect was weak, which is consistent with the *in vitro* results (Fig. 4B). To further explain the dramatic temperature effect when CRISPR-Cas9 is expressed from plasmids, we quantified the sgRNA and the Cas9 protein expression under different culture temperatures. We found that sgRNA expression was up-regulated with increased culture temperature. Culturing at 39°C significantly induced sgRNA expression, up to 6-fold, compared to at 30°C in K562 and Jurkat cells (Figs. 4C and S8A). While in 293T cell, in which the genome editing efficiency is not greatly improved at 39°C in comparison to 37°C, sgRNA expression was not increased at higher temperature (Fig. S8B). Western blot confirmed that there was no significant difference on Cas9 protein expression under different culture temperatures (Fig. 4D). Thus, the increase of CRISPR-Cas9 mediated genome editing obtained from higher temperature resulted, at least in part, from the higher enzyme activity and sgRNA expression.

DISCUSSION

Here, we demonstrated that culture temperature has a significant effect on the efficiency of site-specific nuclease mediated genome editing. While hypothermia treatment enhances ZFN and TALEN performance significantly, it dramatically reduces CRISPR-Cas9 genome editing efficiency. We tested 10 sgRNAs at 9 unique endogenous gene loci and observed the same trend in K562 cells. We further tested several commonly used cell lines and observed similar results.

Intriguingly, we found that 39°C culture can increase CRISPR-Cas9 editing efficiency at various genomic loci in K562 and Jurkat cells. Enhancing genome editing efficiency by culturing cells in 39 °C is simple and allows for robust cell proliferation. While 39°C culture also increased editing at several off-target sites (Fig. S7), the specificity can be improved by designing sgRNAs with minimal potential off-targets or using Cas9 variants with higher fidelity (Kleinstiver et al., 2016; Slaymaker et al., 2016). In other cell lines we tested, we did not observe this enhancement at 39°C, with 37°C culture resulting in the best editing efficiency.

To understand the mechanisms behind our observations, we quantified CRISPR-Cas9 activity both *in vitro* (in test tubes) and *in vivo* (in cultured cells) at different temperatures, and delivered CRISPR-Cas9 via RNP as well as via DNA plasmids. We found that temperature had an effect on both CRISPR-Cas9 enzyme activity and sgRNA expression from plasmids. When RNP was used, gene editing efficiency was slightly higher at 37°C and 39°C (Fig. 4B). When plasmids were transfected into K562 and Jurkat cells, in which we observed higher gene editing efficiency at 39 °C, the sgRNA expression was upregulated at 39°C (Fig. S8A). This effect was not observed in 293T cells, in which the temperature had a minor effect on gene editing (Figs. S4B and S8B). These results suggest that sgRNA expression level is one of the factors determining CRISPR-Cas9 gene editing efficiency. These findings have implications for applying CRISPR-Cas9 editing to organisms that live under different temperatures and for selecting the best delivery format.

We suspect that a similar effect can be observed in other CRISPR systems, such as saCas9 (Ran et al., 2015) and Cpf1 (Zetsche et al., 2015), because they also utilize small RNAs as guide. It would be interesting to test our finding in more cell types, and in combination with other previously reported methods that improve genome editing, to explore whether they can work synergistically.

Our findings indicate that CRISPR-Cas9 responds differently to temperature compared with ZFN and TALEN and may also respond differently to other environmental variables due to its protein-RNA composition.

MATERIALS AND METHODS

Cell culture

All cell culture related products were purchased from Life Technologies, except as specifically noted. K562 and Jurkat (ATCC) cells were cultured using RPMI supplemented with 10% FBS, 1% L-glutamine and 1% penicillin-streptomycin. Cell lines 293T, HepG2 and MCF7 (ATCC) were all cultured in DMEM medium containing 10% FBS, 1% L-glutamine and 1% penicillin-streptomycin. Human ESCs were grown on Matrigel using E8 medium. All cell lines were tested for mycoplasma contamination using nested PCR every two weeks and remained negative.

Construction of plasmids

SgRNAs were designed using CRISPR design webtool (<http://crispr.mit.edu/>) (Table S1). Chemically

synthesized oligos were ordered from Genewiz (China). Two complementary oligos were annealed and then ligated to vector PX330 linearized by *Bbs* I, following standard protocols (Cong et al., 2013).

***In vitro* transcription of sgRNA**

Forward primers containing T7 promoter, 20 bp targeting sequence, and sgRNA backbone forward sequence were used together with sgRNA backbone reverse primer to generate T7-sgRNA DNA fragments using PX330 vector as template in PCR reactions (Table S2). SgRNAs were synthesized by *in vitro* transcription using T7-sgRNA PCR products as templates, using MEGAshortscript T7 kit (ThermoFisher Scientific, USA). Synthesized sgRNAs were purified using MEGAclear kit (ThermoFisher Scientific, USA).

Transfection

All cells were transfected using a LONZA 4D-nucleofector X and corresponding nucleofector kits according to the manufacturer's instructions. Briefly, 5 μ g or 2 μ g of CRISPR-Cas9, TALEN, or ZFN-expressing plasmids were nucleofected into 1×10^6 cells. In RNP electroporation experiments, 5 μ g Cas9 protein (NEB, USA) and 2.5 μ g synthesized sgRNA were mixed and incubated at 37°C for 10 min, then nucleofected using corresponding program (Wang et al., 2016). After transfection, cells were divided into four parts equally, and then incubated at 30°C, 33°C, 37°C, and 39°C independently. After 3 days culture, cells were harvested for further analysis.

Surveyor assay for genome editing efficiency quantification

Surveyor assay was performed as described (Guschin et al., 2010). Genomic DNA was extracted by incubating ten thousand cells with ten microliter lysis buffer (10 mM Tris·Cl, pH 8.0; 2 mM EDTA; 2.5% (Vol/Vol) Triton-X 100; 2.5% (Vol/Vol) Tween-20; 100 ng/mL Proteinase K), and incubated at 50°C for 60 min, followed by 95 °C for 15 min. 1 μ L of lysate was used in a 25 μ L PCR reaction using AccuPrime Taq polymerase (Invitrogen, USA) (Table S2), and the PCR conditions are as follows: 94°C for 2 min; 30X (94°C for 20 s, 60°C for 20 s, 68°C for 40 s); 68°C for 3 min; hold at 4 °C. 6.5 μ L PCR products were then denatured, and annealed with 3 μ L 1X AccuPrime Buffer II, then digested with 0.5 μ L Surveyor nuclease (Integrated DNA Technologies, IDT, USA). Samples were run on 10% acrylamide TBE gel, stained with ethidium bromide for 10 min, rinsed with water and then exposed on

Bio-rad gel imager. The band intensities were quantified using Image J software, and the genome editing efficiency was calculated using the equation: % genome editing = $100 \times (1 - (1 - \text{fraction cleaved})^{1/2})$ (Guschin et al., 2010).

RFLP assay

Target DNA fragments were amplified by PCR and purified using QIAquick PCR purification kit(QIAGEN, Germany). 200 ng purified DNA was digested by 10 units of corresponding restriction endonuclease (NEB, USA) at 37 °C for 2 h. Reaction mixtures were run on 15% TBE acrylamide gels and stained with ethidium bromide. The band intensities were quantified using Image J software. HDR efficiency was calculated using the equation:(b + c) / (a + b + c) × 100%, where “b” and “c” indicate the band intensity of the two cleaved bands, and “a” represents the full length band intensity. Genotyping primers were listed in Table S2.

TIDE analysis

For each sample, target region was PCR amplified, purified and Sanger-sequenced. Each sequence chromatogram was analyzed using TIDE webtool (<http://tide.nki.nl>).For analyzing gene modification frequencies, sequence chromatogram from an un-transfected control sample was used as a reference sequence. Default parameters were used for all analysis. The HDR frequencies at *OCT4* and *MECP2* loci were calculated as the +1 nt insertion frequency of donor samples minus the basal +1 nt insertion frequencies of no-donor samples.

In vitro cleavage assay

Mouse *Tet2* fragment was amplified by PCR and cloned into pEASY-T1 cloning vector (Transgen, Beijing, China). Restriction enzyme XmnI (NEB, USA) was used to linearize the vector. Linearized plasmid (500 ng) was incubated with Cas9 protein (300 ng) and sgRNA (300 ng) in 15uL Cas9 buffer(1X)for 10 min or 60 min at 30°C, 33°C, 37°C or 39°C. After incubation, DNA was purified by phenol:chloroform extraction and ethanol precipitation. Purified DNA samples were run on 1% agarose gel and stained with ethidium bromide.

Quantitative real-time RT-PCR

RNA was extracted using the RNeasy Mini Kit (Qiagen, Germany). cDNA was reverse transcribed using TransScript miRNA First-Strand cDNA Synthesis SuperMix (Transgen, Beijing, China). For detecting *AAVS1* and *MECP2* sgRNA expression, we designed primers on the 20 nt protospacer sequence (Table S2). All reactions were run on Roche LightCycler 480. SgRNA expression was determined relative to *U6* snoRNA using the $\Delta\Delta Ct$ relative quantification method.

Western blot analysis

K562 Cells were collected 3 days after nucleofection. Equal number of cells were lysed in cold RIPA buffer containing protease inhibitor cocktail for 5 min on ice (Thermo Scientific, USA) and subjected to SDS-PAGE and standard Western blot analysis. Anti-Flag or GAPDH primary antibodies were used. SuperSignal WestPico Chemiluminescent Substrate (Thermo Scientific, USA) and the ChemiDoc MP Imaging System (Bio-Rad, USA) are used for visualization.

Statistical analysis

All statistical analyses were performed using GraphPad Prism 5 (GraphPad Software Inc, La Jolla, CA, USA). The data was represented as mean \pm standard deviation (SD). The *P* values were determined by Student's *t*-test (unpaired).

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FIGURE LEGENDS

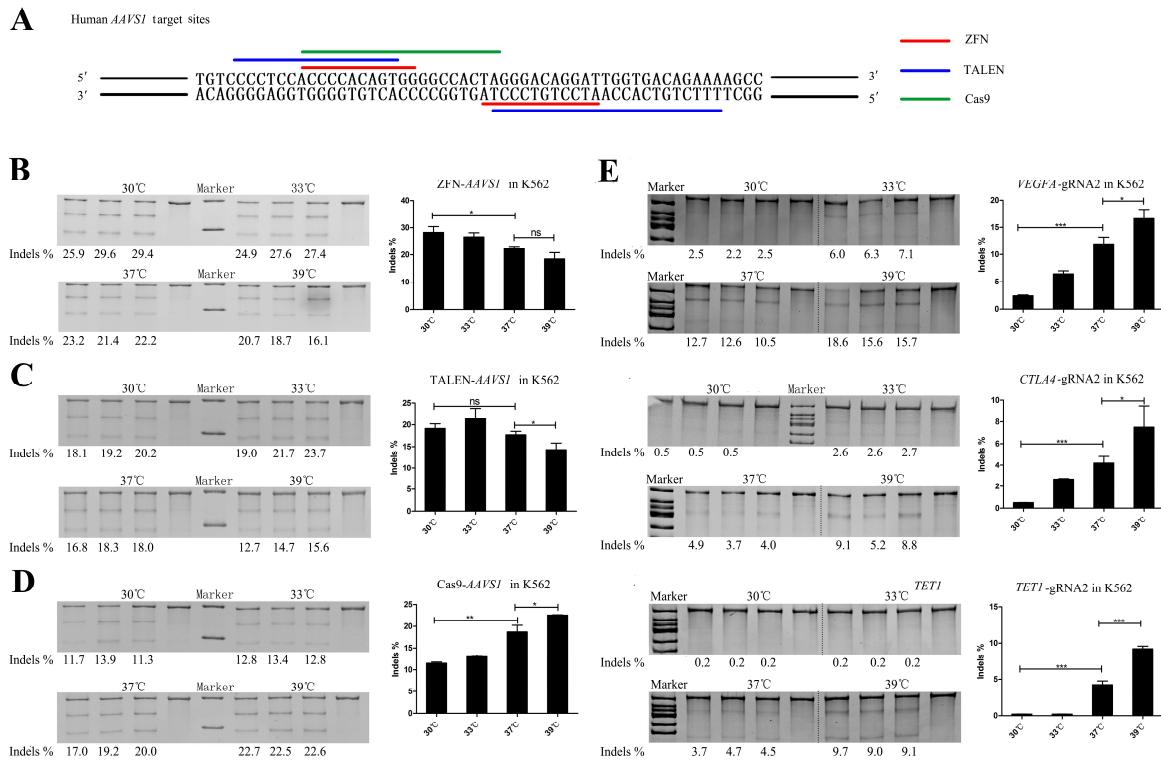
Fig. 1. The temperature effect on ZFN, TALEN and CRISPR-Cas9 mediated genome editing. **A:** Schematic of ZFN, TALEN and CRISPR target sites at the human *AAVS1* locus. Surveyor assay to determine the editing efficiency of ZFN (**B**), TALEN (**C**), CRISPR-Cas9 (**D**) at human *AAVS1* locus. **E:** Surveyor assay to determine the CRISPR-Cas9 editing efficiency at *VEGFA*, *CTLA4*, and *TET1* loci in K562 cells. Experiments were performed in three biological replicates, and error bars indicate SD. On the gel, each temperature had 4 samples, the left three are experimental samples, and the right one is wild-type control.

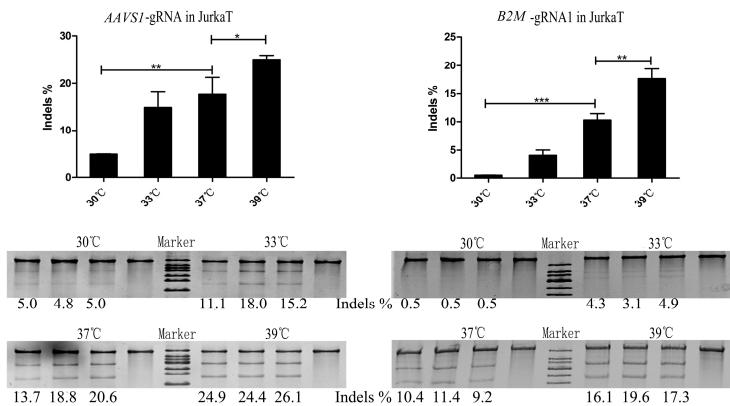
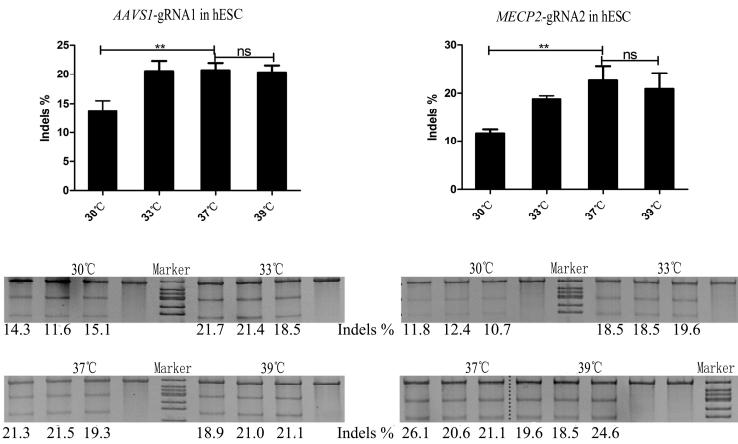
Fig. 2. Temperature effect on CRISPR-Cas9 mediated genome editing in Jurkat cells and human ESCs. **A:** Jurkat cells; **B:** human ESCs. Surveyor assays were performed in three biological replicates, and error bars indicate SD. On the gel, each temperature had 4 samples, the left three are experimental samples, and the right one is wild-type control. For *MECP2* in human ESC, the two control samples are located at the right side of the gel.

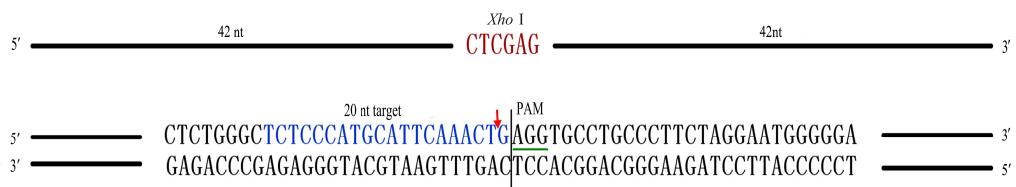
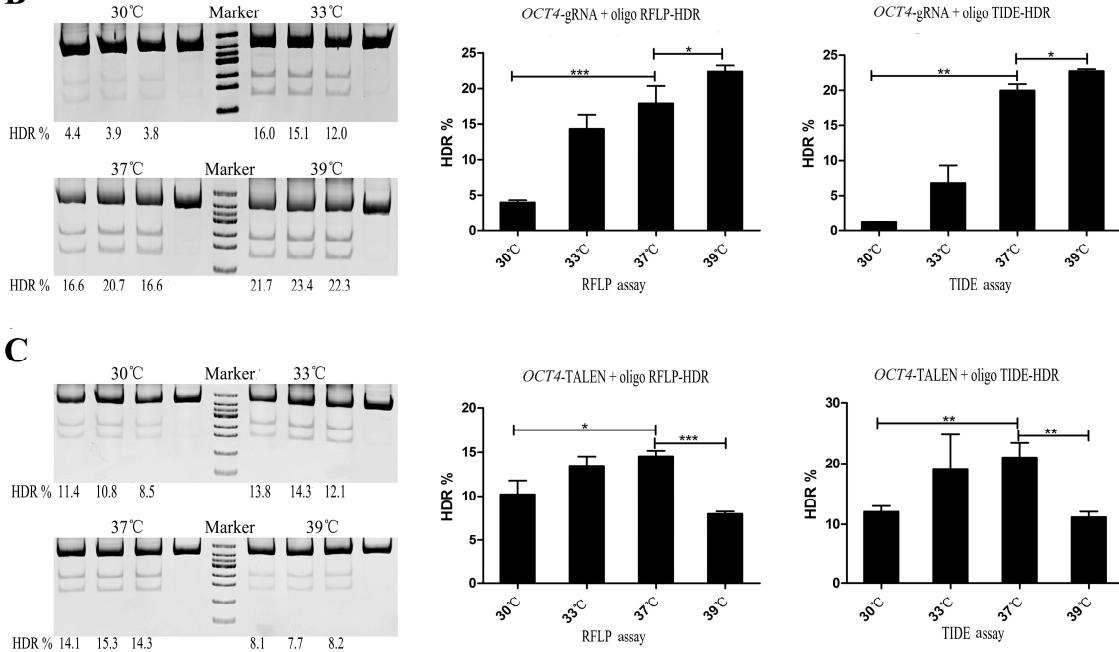
Fig. 3. Temperature effect on CRISPR-Cas9 and TALEN mediated HDR editing efficiency. **A:** Schematic of human *OCT4* target site and HDR single-strand oligo donor. Red arrowhead indicates the

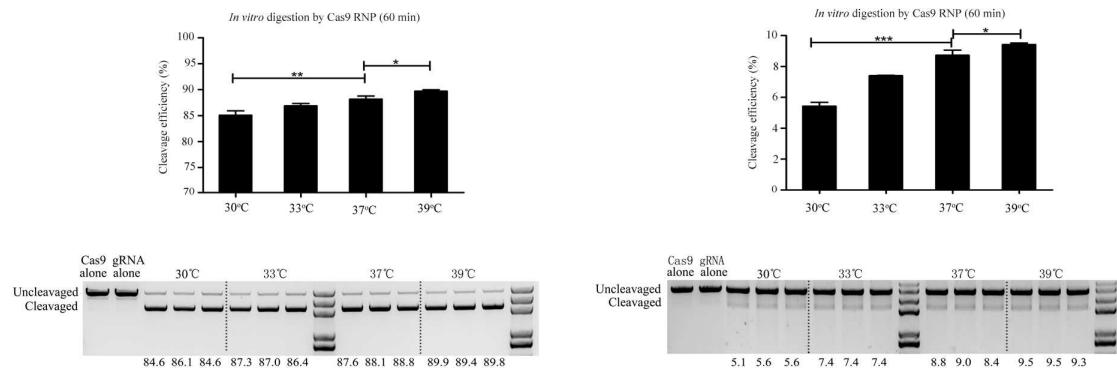
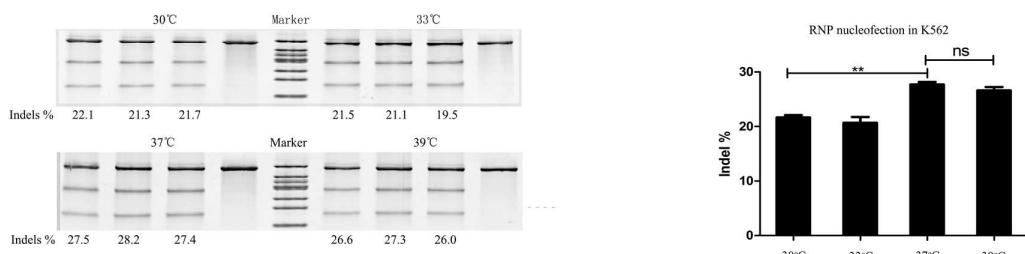
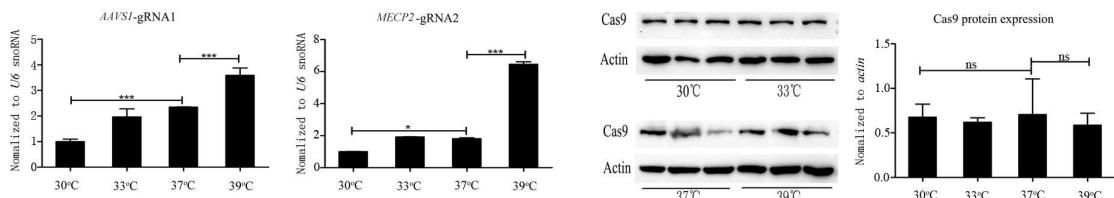
site of one base insertion. PAM is labeled by green underline. **B:** RFLP assay and TIDE assay of CRISPR mediated HDR repair. **C:** RFLP assay and TIDE assay of TALEN mediated HDR repair. Experiments were performed in three biological replicates, and error bars indicate SD.

Fig. 4. The mechanisms of temperature effect on CRISPR-Cas9 mediated genome editing. **A:** Cas9 RNP enzymatic activity *in vitro*. The left panel: samples digested at room temperature for 60 min; the right panel: samples digested at room temperature for 10 min. **B:** Surveyor assay to determine Cas9 RNP mediated genome editing efficiency at *AAVS1* locus in K562 cells. **C:** Quantitative real-time RT-PCR to determine sgRNA expression in K562 cells. Data were normalized to U6 snoRNA. **D:** Western blot analysis of the Cas9 protein expression at different temperatures. All experiments were performed in three biological replicates, and error bars indicate SD.



A**B**

AHuman *OCT4* target site**B**

A**B****C****D**