High Fidelity CRISPR-Cas9 reagents for stem cell editing

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Abstract

CRISPR/Cas9 has become one of the most used gene therapy tools due to its simplicity and effectiveness. To improve the usefulness of the method for clinical and RUO applications, improvements to increase on-target editing efficiency and homology-directed repair (HDR) as well as reducing undesired off-target genome cuts are needed. We applied a NGS assay, TEG-seq (1) to screen several in-house engineered and published high-fidelity (HF) Cas9 variants. We identified a HF-Cas9 variant (HF-Cas9v4), which outperformed others for improved specificity and functional activity. To further improve the on-target editing efficiency, especially insertion of SNPs or genes by HDR, we screened dozens of candidates that modulate DNA repair pathways. One candidate dramatically improves HF-Cas9 on-target HDR efficiency through the mechanism of extending and maintaining the single strand overhang at the double strand break site, forcing the cellular repair to favor HDR. We found that this enhancement of on-target activity does not increase off-target mutations.

By using the HF-Cas9 and our enhancer we demonstrated this non-viral system can edit iPSC with high on-target and HDR efficiency while maintaining low off-targets on the sickle cell disease related HBB SNP site, and other genes (TRAC, TRBC, CD52, PD1) related to CAR-T cancer therapy. The enhancements to the CRISPR-Cas9 toolset will significantly benefit gene therapeutic research and clinical trials

Introduction

- CRISPR/Cas9 has become one of the most used gene editing and therapy tools due to its simplicity and effectiveness
- Improvements to increase on-target editing efficiency, especially homology-directed repair (HDR) while reducing undesired off-target genome cuts are needed
- We screened and identified HF-Cas9 (HF-Cas9v4) and editing enhancer that significantly increased editing specificity and activity, especially HDR.

Conclusions

- Using a genome-wide off-target screening assay TEG-seq. we identified a HF-Cas9 variant (HF-Cas9v4) that outperformed other HF-Cas9 including HF-Cas9v3 from a commercial supplier for improved specificity (Figure 1, 2, 4 and Table 1)
- We also screened and identified genome editing enhancer that dramatically improved on-target HDR efficiency through the mechanism of extending and maintaining the single strand overhang at the double strand break site, forcing the cellular repair to favor HDR (Figure 3, 5 and Table 1)
- In T cell, HF-Cas9v4 with enhancer showed ~50% HDR in average from 23 gRNAs targeting CD52, TRAC, TRBC and PD1 genes with lower or no detectable off-target compared to wt-Cas9 and HF-Cas9v3 (Table 1)
- In iPSC, HF-Cas9v4/gRNAs targeting two SNPs on hemoglobin β subunit gene (HBB) and BCL11A related to Sickle Cell disease showed significantly lower off-targets compared to wt-Cas9 and HFv3 Cas9
- The genome editing enhancer does not increase off-target (data not shown)

Results

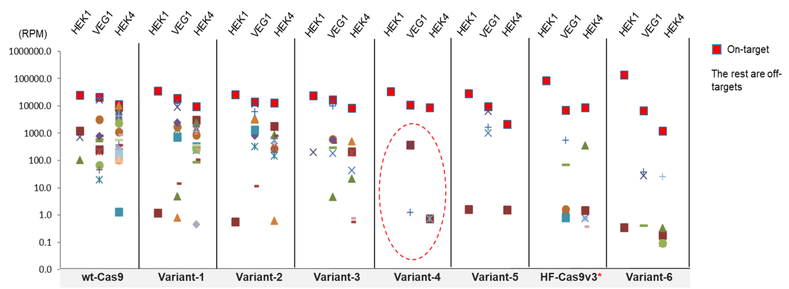

Figure 1. Genome-wide off-target screening in HEK293 cell using TEG-seq: RPM (Reads Per Million) were plotted from different Cas9 variants RNP edited samples for HEK1, VEG1 and HEK4. Variant-4, -5, and -6 contained less off-targets compared to the controls of wt-Cas9 and HF-Cas9v3. Variant-4 created the least off-target.

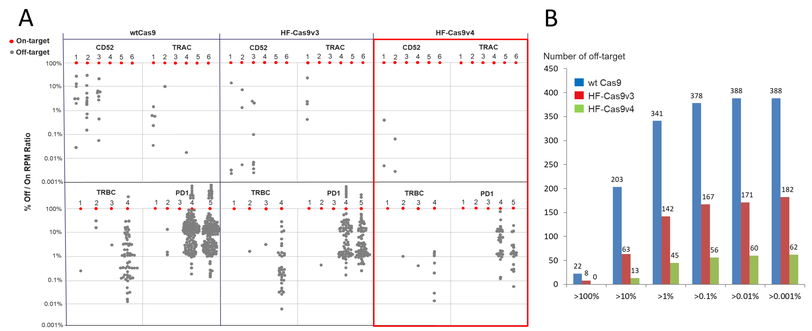

Figure 2: Off-target detected on 21 gRNAs targeting four CAR-T related therapeutic genes edited by three Cas9 RNP in T cell: (**A**) RPM Ratio (%) of off/on-targets were plotted based on RPM (reads per million) from TEG-seq. All the RPMs from off-targets were normalized relative to the RPM of on-target (100%). Thus, each off-target was given a probability at % scale relative to the corresponding on-target. Red dots are on-targets, gray dots are off-targets. HF-Cas9v4 generated much fewer off-targets on 21 gRNAs targeting CD52, TRAC, TRBC and PD1. (**B**) Number of off-targets detected at different probability scales relative to on-target was plotted. HF-Cas9v4 generated fewer off-targets in all probability scales. More importantly, it created fewer (13) off-targets at high probability scale (>10%), compared to wt-Cas9 (203) and HF-Cas9v3 (63).

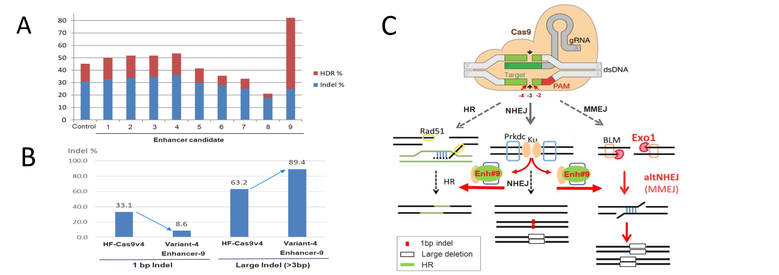

Figure 3. Screening and identification of genome editing enhancer in T cell: (**A**) Nine enhancers were screened for their ability to improve HDR editing efficiency in T cells. One of enhancer (Enh#9) dramatically increased HDR activity. (**B**) Deep sequencing analysis indicated Enh#9 dramatically decreased 1bp indel that is one of major repair pattern from NHEJ. It increased large Indel and showed 2-6 micro homolog bases at repair site in most (>90%) of reads, indicated high MMEJ (data not shown). **(C)** Possible mechanism of Enh#9 enhances MMEJ and HDR. Enh#9 may bind to and pull away Ku and DNA-PK at DSB sites that leads to more DSB ends expose to exonuclease and creates longer or more single strand overhang, provides more probability of micro-homology coupling (MMEJ) between two strands. If donor DNA exists, longer or more single strand overhang at DSB provides more probability for micro-homology coupling for HR, or expose to Rad51 that recruits other proteins for HR repair complex and results in higher HR

Table 1. Summary of on-target, off-target and HDR on 23 gRNAs targeting CAR-T related therapeutic genes using three Cas9 and HF-Cas9v4 plus enhancer in T cell: By using HF-Cas9v4 and enhancer, ~50% HDR can be achieved in T cell in edited CD52, TRAC, TRBC and PD1 with no detectable off-target

	% On-target cleavage			Off-target detected (Off/On>0.001%)			% HDR (ssDonor)			
gRNA	wt-Cas9	HF-Cas9v3	HF-Cas9v4	wt-Cas9	HF-Cas9v3	HF-Cas9v4	wt-Cas9	HF-Cas9v3	HF-Cas9v4	HFCas9v4+E
CD52_1	77.47	56.37	54.27	7	3	2				
CD52_2	79.36	72.69	76.89	11	3	2				
CD52_3	83.61	73.79	73.01	9	8	0				
CD52_4	71.58	60.50	61.28	0	0	0	41.83	42.95	41.13	74.99
CD52_5	80.24	70.34	76.95	0	0	0	35.67	34.39	35.25	75.17
CD52_6	86.42	60.89	35.76	0	0	0	49.49	51.27	38.43	40.45
TRAC_0	71.10	38.44	37.77	0	0	0	30.08	16.38	17.37	36.06
TRAC_1	12.21	11.06	11.04	5	5	0				
TRAC_2	67.25	59.08	55.54	1	0	0	21.75	19.95	15.79	29.95
TRAC_3	9.83	11.56	5.20	0	0	0				
TRAC_4	92.12	88.90	89.60	1	0	0	20.93	22.79	17.41	75.07
TRAC_5	39.49	19.62	24.17	0	0	0				
TRAC_6	53.82	33.14	45.19	0	0	0	4.70	3.04	6.53	30.91
TRBC_1	66.45	56.02	66.97	1	0	0	23.14	18.12	23.29	59.15
TRBC_2	17.48	14.58	13.72	2	1	1	29.24	21.17	16.35	58.08
TRBC_3	7.78	3.59	3.38	1	1	1				
TRBC_4	59.39	46.16	53.18	52	31	7				
TRBC_5	21.57	19.69	19.23							
PDCD1_1	48.30	19.19	41.17	0	0	0	4.63	2.92	3.23	29.42
PDCD1_2	61.30	23.74	61.47	3	1	0	14.94	9.54	16.23	45.32
PDCD1_3	33.52	17.61	26.41	0	0	0	3.77	0.97	2.54	39.71
PDCD1_4	4.83	4.89	5.12	155	67	29				
PDCD1_5	14.69	10.33	18.07	134	62	20				
Average	50.43	37.92	41.54	17.36	8.27	2.82	23.35	20.29	19.46	49.52

HBB1			Reads Per Million (RPM)		
Target	Align_sequence	PAM	wt-Cas9	HF-Cas9v3	HF-Cas9v4
On	CTTGCCCCACAGGGCAGTAA	CGG	141922	258580	284917
Off1	TCA..............	GGG	126583	970	132
Off2	T.............T.G.	CAG	13100	15871	1229

HBB2			Reads Per Million (RPM)		
Target	Aligned_sequence	PAM	wt-Cas9	HF-Cas9v3	HF-Cas9v4
On	CATGGTGCATCTGACTCCTG	AGG	431212	356556	452904
Off1	...AA..............	TGG	246927	3153	319
Off2	..A..A....C.....C....	GGG	929	228	7
Off3	G..............A.	AGG	118	3197	0

HEK4			Reads Per Million (RPM)		
Target	Align_sequence	PAM	wt-Cas9	HF-Cas9v3	HF-Cas9v4
On	GGCACTGCGGCTGGAGGTGG	GGG	82924	174851	159782
Off1	...G.....G........	AGG	149096	84883	8695
Off2	.....GA..........	GGG	151950	390	105
Off3	A.G..........G.....	TGG	246887	927	124
Off4	.......T.....C.....	AGG	118633	119	65
Off5	....A.........C.C..	AGG	12949	8	0
Off6	..........G.....C..	GGG	3005	21	0
Off7	T..........C.....A..	TGG	1734	0	0
Off8	....-G.....g........	AGG	99	0	0
Off9	...T.............G..	TGG	52	0	0

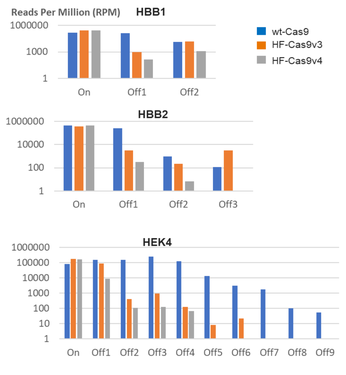

Figure 4: Off-target detected in iPSC on two gRNAs targeting two SNPs on hemoglobin β subunit gene (HBB) related to Sickle Cell disease and HEK4, a well-studied site for off-targets in different cells: (Left) showed PRM (reads per million) for each on- and off-targets. (**Right**) showed graph bars for the off-target detected. HF-Cas9v4 showed higher fidelity compared to wt-Cas9 and HF-Cas9v3. (**note**: TEG-seq didn't detect any off-target on BCL11A gRNA (data not shown).

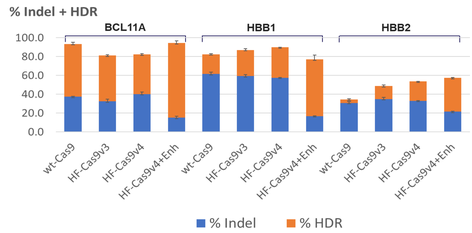

Figure 5: On-target activity (Indel and HDR) in iPSC using HF-Cas9v4 and enhance with three gRNAs targeting two SNPs on hemoglobin β subunit gene (HBB) and BCL11A related to Sickle Cell disease. The HF-Cas9v4 remained high on-target activity and reached to ~50% HDR with enhancer.

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References

1. Pei-Zhong Tang , Bo Ding, Lansha Peng, Vadim Mozhayskiy, Jason Potter, Jonathan D Chesnut. TEG-seq: an ion torrent-adapted NGS workflow for *in cellulo* mapping of CRISPR specificity. ***Biotechniques***. 2018, 65(5): 259-267.