Examining relationships between on-target efficiency and specificity of ZFNs, TALENs, and CRISPR/Cas9 on editing the CCR5 gene

Hypothesis: The use of CRISPR, TALENs, and ZFNs separately show that CRISPR provides the benefits over the other techniques.

Abstract

Human immunodeficiency virus and acquired immunodeficiency syndrome (HIV/AIDS) is a spectrum of conditions that result from infection with HIV. C-C chemokine receptor type 5 (CCR5), is a protein on the surface of white blood cells involved in the immune system. Certain individuals carry a mutation, CCR5-Δ32, protecting them against HIV. Treatment for AIDS-related illness has been very effective, but is neither a prophylactic nor a cure. Using gene editing methods, CCR5 can be artificially mutated to cause CCR5-Δ32; However, most approaches to CCR5 inhibition aim solely to edit CCR5. There are three current possible methods to mutagenize the CCR5 gene: Zinc finger proteins (ZFNs), Transcription activator-like effector nucleases (TALENs), and Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR associated protein 9 (CRISPR/Cas9).

The goal of this study is to evaluate the potential of these three techniques in editing the CCR5 coreceptor on different cell systems. A systematic literature search using databases was pursued. Data was combined in a table and meta-analysis of these findings was performed. When comparing efficiency and specificity of the three-independent methods, CRISPR/Cas9 was identified as the optimal method to edit CCR5. Understanding which genetic engineering tool is the most efficient could help researchers focus on the best method to improve the way patients with HIV are treated and/or potentially cure them.

Introduction

Since 1981 35.0 million of people have died from AIDS-related diseases. HIV is a retrovirus with a single strand of RNA that, once inside cells, uses its own reverse transcriptase to encode DNA. Transmission of the virus can be sexual, by contact or transfer of blood, or non-sexual, through pregnancies or breastmilk.

An early step in infection by HIV-1 requires collaboration between CD4 and coreceptors on surfaces of susceptible cells (Figure 1).[1] The major coreceptor for macrophage-tropic isolates of HIV-1 is CCR5, expressed on white blood cells associated T cell.[2]

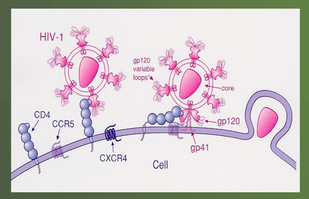

Figure 1. Chemokine-HIV attachment process. Includes: (1) attachment from the gp120 loop to the CD4+ receptor, (2) attachment of a variable from the gp120 to a co-receptor CCR5 and (3) fixation of the gp41 to the cellular membrane, and invading of a HIV virion into the cell

10% of North American Caucasians carry the CCR5 Delta 32 mutation, resulting in a premature stop and a truncated, putatively-defective protein (Δ32).[3] The resistance of Δ32/Δ32 homozygous individuals to infection by HIV-1 strongly suggests that CCR5-tropic viruses are critical for viral transmission (R5 tropism).[4] Using gene editing methods, CCR5 can be artificially mutated to phenocopy, or reproduce, CCR5Δ32.[5] However, most approaches to CCR5 inhibition aim solely to knockout CCR5.

There are three widely-used methods to mutagenize the CCR5 gene. ZFNs have been designed to recognize all of the 64 possible trinucleotide combinations, and ZFNs can recognize nearly any specific sequence of DNA triplets (Figure 2A).[6] TALENS recognizes single nucleotides instead of triplets, and when combined to a nuclease , DNA can be cut at specific locations (Figure 2B).[7]

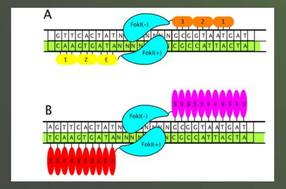

Figure 2. (A) ZFN recognizes triplets and the FokI nuclease operates as a dimer, cutting in the spacer region between two distinct ZF target sites. TALEN, similar in principle to the ZF nuclease, the components of the array recognize individual nucleotides.

CRISPR-Cas9 and other systems can be programmed to target specific stretches of genetic code and to edit DNA at precise locations.[8] The Cas9 system (Figure 3) works by "cutting" the specific 20 bp ('') viral/DNA trait out using an enzyme to create a double-stranded break (DSB), and guides and replaces the gene with a desired gene (Zhang, Wen, & Guo, 2014). The CRISPR/Cas systems consist of a Cas9 endonuclease that is directed to cleave a target sequence by a guide RNA (sgRNA). Specificity is conferred through the crRNA and function requires a tracrRNA Figure 3). [9]

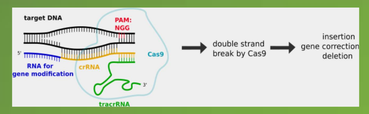

Figure 3. The sgRNA identifies the target sequence which is cleaved by a Cas9 endonuclease. The resulting double stranded break allows the RNA for gene modification to be placed into the target sequence.

If ZFNs, TALENs or CRISPR/Cas9 bind at a non-target sequence, a single or double strand break can be induced and cause non-specific and unintended point mutations, deletions, insertions, inversions, and translocations.[10] Tools to detect off targets effects include: screens *in situ* along with targeted, exome, and whole genome sequencing. The biased approach will only examine the intended area of capture, while unbiased detection of off-target effects remains difficult, genome-wide methods enable careful, empirical consideration of possible alterations.

The goal of this study is to evaluate the potential ZFNs, TALENs, and CRISPR/Cas9, in editing the CCR5 HIV coreceptor on T cells. Understanding which genetic engineering tool is the most efficient could help researchers focus on the best method to improve the way patients with HIV are treated.

Methods

Sample of Studies

Reports related to CCR5 mutagenesis using ZFN, TALENs and CRISPR/cas9 that were available by February 2018 were retrieved. EBSCOhost (1998-2018), Nature (2003-2018), National Center for Biotechnology Information (2003-2018), Cell (2005-2018), Public Library of Science (2005-2018), JSTOR (2008-2018), and Elsevier (2011-2018) as well as specific journals were searched, using the keywords, zinc finger nucleases, TALENs, CRISPR, CCR5 and mutagenesis, genetic engineering, CCR5 and HIV (-1), off-target mutations, CRISPR and CCR5, CCR5 knockout, TALENs and CCR5, ZFNs and CCR5, and HIV tropism.

Selection Criteria of the studies included

1. Genetic engineering of CCR5
2. ZFN, TALENs and CRISPR/cas9 mutagenesis techniques
3. Performed in cell lines or mammalians species
4. Reported levels of CCR5 gene editing
5. Off target effects when stated

23 data sets reported in 16 independent reports were found.

Moderators

For descriptive purposes, the year and the source were recorded for each report. Characteristics of the individual studies are presented in Table 1. The studies often contained several independent datasets such as different measures and different methods.

Dependent Measures and Statistical Analysis

The type of cells, the nuclease used, the efficiency of editing, the method of transfection used for gene editing, the off targets effect, the predicted gene as well as the references where the data were reported represent the dependent measures. Data were analyzed by averaging each data point and by calculating standard error of the mean. Data was graphed using GraphPad Prism software and differences among multiple means were tested by one-way ANOVA followed by a Fisher-test using Statview software.

Results

Disruption of the CCR5 gene

The individual percent of efficiency for CCR5 gene disruption using ZFNs, TALENs and CRISPR/cas9 found in each peer reviewed paper are reported in Table 1. After systemic search of the different databases listed in the method section, 16 manuscripts corresponded to the described selection criterion to enter the study (Table 1).

Out of the selected manuscripts, a range of cell systems had been used: hematopoietic stem progenitor cells (HSPC), induced pluripotent stem cells (iPSC), human embryonic stem cells (hESCs), peripheral blood mononuclear cells (PBMCs), HeLa cells expressing CD4 receptors (HeLa-CD4), chronic myelogenous leukemia cells (K562), HeLa cells expressing CD4, CCR5, and CXCR4 (T and human embryonic kidney (HEK293T). The number of variants and method of transfection varied amongst the manuscripts (Table 1).

ZFNs edited on average the CCR5 gene by 23.4%, TALENs by 19.9 % and CRISPR/Cas9 by 37.3% (Figure 4). CRISPR/Cas9 showed statistically significantly higher efficiency than ZFNs and TALENs (p=0.020 and p=0.031 respectively).

Off Target Results

All three techniques were compared by their off-target effects. Of the detected off target deletions, CRISPR was found to only have one case of a 9 bp insertion or deletion of bases in the genome (indel) in the CCR2 gene, whereas TALEN had 0.12, 0.31, and 8.16% identified edits in three data sets on the predicted gene of CCR2. Two data sets detected off target knockouts by ZFNs, editing 5.39 and 11% of the predicted CCR2 gene respectively. Out of the three-tested techniques, CCR2 was the gene that was biasedly tested for off target effects.(Table 1).

Table 1. ZFNs, TALENs, and CRISPR/Cas9-mediated CCR5 editing in human cells

Study Analyzed	Cell Type	Nucleases	Efficiency (%)	Method of Transfection	Off Targets (OT)
Yu et al. (2017)	iPSCs	ZFN	10.9	Lipofectamine	none detected
Didigu et al. (2014)	HEK293T	ZFN	6	Lentiviral vectors	none detected
Didigu et al. (2014)	HEK293T	ZFN	13.5	Lentiviral vectors	none detected
Didigu et al. (2014)	HEK293T	ZFN	24	Lentiviral vectors	none detected
Mussolino et al. (2014)	HEK293T	ZFN	14	Polyethylenimin (PEI)	11% CCR2
Lei et al. (2011)	hESCs	ZFN	36	Lentiviral vectors	N/A
Holt et al. (2010)	HSPCs	ZFN	17	Nucleofector	N/A
Perez et al. (2012)	PBMCs	ZFN	58	Lentiviral vectors	5.39% CCR2
Li et al. (2013)	HSPC	ZFN	31.3	Lentiviral vectors	none detected
Yu et al. (2017)	HeLa-CD4	TALEN	15.4	Lipofectamine	none detected
Mock et al. (2015)	PBMCs	TALEN	19.4	Lentiviral vectors	0.12% CCR2
Mock et al. (2015)	PBMCs	TALEN	32.8	Lentiviral vectors	0.31% CCR2
Miller et al. (2010)	HEK293T	TALEN	21	Nucleofector	N/A
Mussolino et al. (2014)	K562	TALEN	17	Nucleofector	8.16% CCR2
Ye et al. (2014)	iSPCs	TALEN	14	Lentiviral vectors	none detected
Yu et al. (2017)	HeLa-CD4	CRISPR	20.7	Polyethylenimin (PEI)	none detected
Dang et al. (2015)	TZM-bl	CRISPR	55.9	Lipofectamine	N/A
Liu et al. (2017)	PBMCs	CRISPR	40.6	Lentiviral vectors	none detected
Wang et al. (2014)	TZM-bl	CRISPR	42.5	Lentiviral vectors	9 OT
Kang et al. (2015)	iPSC	CRISPR	42.8	Nucleofector	none detected
Kang et al. (2015)	iPSC	CRISPR	30.8	Nucleofector	none detected
Woo Cho et al. (2013)	HEK293T	CRISPR	33	Nucleofector	none detected
Ye et al. (2014)	iPSCs	CRISPR	33	Lentiviral vectors	none detected

[Note]: References for the studies analyzed can be found in the References section of the Academic Paper

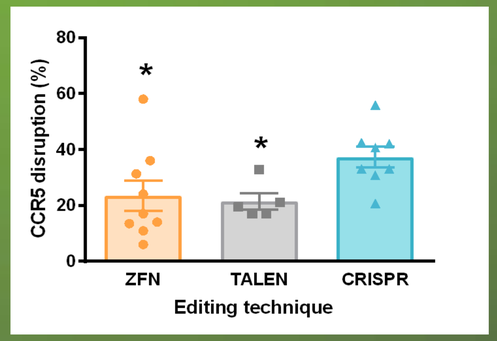

Figure 4. % disruption ot the CCR5 gene was quantified after gene editing with ZFN, Talen and CRISPR techniques were applied. *p<0.05 using one-way ANOVA followed by a Fisher-test.

Discussion

As expected each nuclease edited the CCR5 gene with varying degrees of success. ZFNs averaged the same percent edits as TALENs, with the only such discrepancy being that more off targets were identified in the ZFN-associated studies. As in Ye et al., CRISPR averaged a much higher percent edits of CCR5 than the other techniques.[11] There was a minor off target indel (9") within the CRISPR studies, whereas none were detected otherwise. The lack of detected off target effects suggests that methods for unbiasedly detecting off targets are primitive and rarely used throughout the scientific community. In order to grasp the extent of genome editing, off target detection must be standardized.

While CCR5 is the major co-receptor by which HIV infects cells, it is not the only such co-receptor. It is possible that under selective pressure HIV will evolve to use another co-receptor. However, examination of viral resistance to AD101, the molecular antagonist of CCR5, indicated that resistant viruses did not switch to another coreceptor (CXCR4) but persisted in using CCR5.[2]

Several problems arise in meta-analysis: parameters are often multiple; cell types and methods were different; the MOI was not always identical from one manuscript to the other; not well designed or executed studies may have been included; the data summarized may not be homogeneous; grouping different causal factors may lead to meaningless estimates of effects; and the theory-directed approach may obscure discrepancies.

In order for gene editing technologies to make the leap towards safe and widespread use in the clinic, the rate of off-target modification needs to be measured and minimized and the efficiency of specific editing of the gene of interest must improve.

Conclusion

Since the discovery of CRISPR as a gene editing tool in 2012 it has evolved into a widely-used tool. This study demonstrates that CRISPR represents a step forward as an improvement upon existing technologies. This realization could lead to a clinical breakthrough in HIV/AIDS treatment where an optimized CRISPR molecule would be able to eradicate HIV, instead of simply managing the disease with expensive AIDs therapies. CRISPR methodology for CCR5 editing could be applied to humans in the near future when safety and effectiveness are proven in clinical trials.

Further Work

Genetic engineering is rapidly emerging as a technology that can fight human disease and improve quality of life. From this paper it was concluded that the CRISPR methodology was the most efficient genetic engineering tool, so naturally the next step should be to implement CRISPR against HIV-1 and other viruses if it shows such potential. This topic should be investigated further in head-to-head testing to apply this form of comparison to other viruses and diseases, or even completely different applications of these technologies in order to advance research in this field.

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