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HumBio 158G
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Genome Editing Techniques for Treatment of HIV

We live in an exciting time. Knowledge of the human genome increases yearly at an exponential rate, vastly exceeding the already-extraordinary limit of Moore's Law. The sheer vastness of the data now on-hand, and the largely open means of its global access has ensured consistent innovation worldwide.

As our dexterity with the manipulation of genetic material grows, we begin to search after the applications for our newfound tools. Recently, clinical focus has been centering on combating nature's own genomic editors: retroviruses, and specifically, HIV. This virus has been so devastating precisely due to its ability to modify our genomes, hiding in the one place we, until recently, were unable to get to it – among our own genes. Recently, however, new technologies have emerged suggesting that a cure may be closer than we think. Their applications can be separated into two camps: editing of human immune receptors, and the removal/inactivation of retroviral DNA. In this paper, I discuss the foremost among these technologies, their status with respect to clinical use, and their implications for future treatment.

HIV Infection: Viral Entry and Genomic Integration

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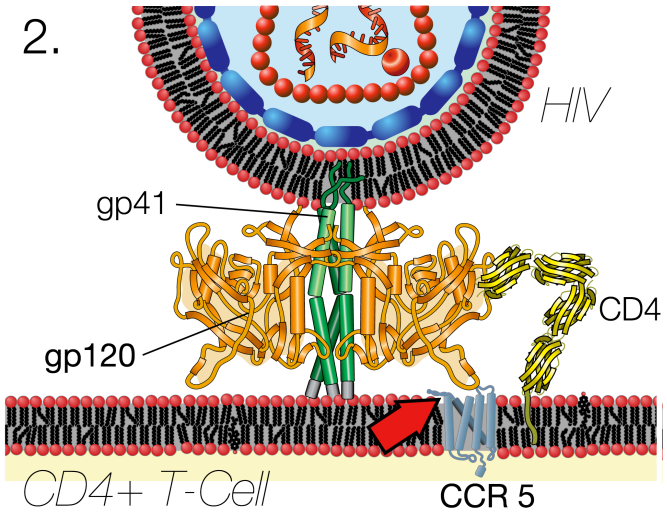

The role of CCR5 in HIV membrane fusion.[1]

HIV is an enveloped, single-stranded positive-sense RNA virus of the family Retroviridae.[2] The viral tropogen, gp120, binds the glycoprotein CD4 and the transmembrane chemokine receptors CCR5 and CXCR4, which coexist in the lipid membrane of immune T-cells.[3] Following membrane fusion, viral reverse transcriptase translates the RNA genome into a double-stranded DNA strand, which is transported to the nucleus and integrated into the human genome by integrase enzyme.[4] The reverse transcription process is highly error prone,[5] and as such generational iterations of HIV virus vary to the point of making gp120 vaccines (or other viral protein vaccines, for that matter) infeasible. While attack on the viral metabolism is thus ruled out, the constancy of the human receptors involved in viral fusion and the relative conservation of HIV

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[1] https://upload.wikimedia.org/wikipedia/commons/6/61/HIV_Membrane_fusion_panel.svg

[2] http://viralzone.expasy.org/viralzone/all_by_species/7.html

[3] Tatjana Dragic, "An Overview of the Determinants of CCR5 and CXCR4 Co-Receptor Function," *Journal of General Virology* 82, no. 8 (August 1, 2001): 1807–14.

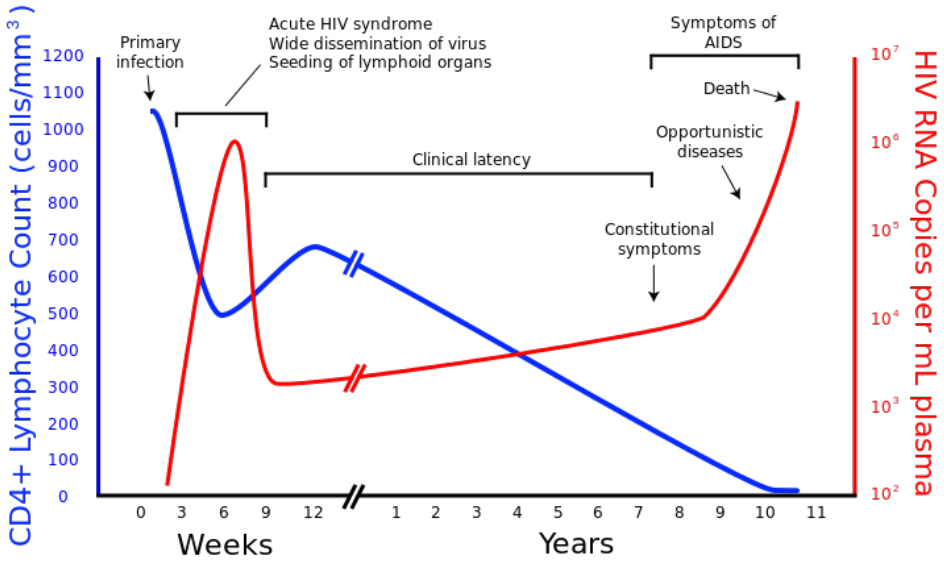
[4] David M. Knipe and Peter M. Howley, eds., *Fields Virology*, 6th ed (Philadelphia, PA: Wolters Kluwer/Lippincott Williams & Wilkins Health, 2013), 1574.

[5] K. Bebenek et al., "Error-Prone Polymerization by HIV-1 Reverse Transcriptase. Contribution of Template-Primer Misalignment, Miscoding, and Termination Probability to Mutational Hot Spots," *The Journal of Biological Chemistry* 268, no. 14 (May 15, 1993): 10324–34.

LTRs (inferred from the high conservation of associated Tat transcription factors)[6] provide targets for genetic knockout and excision.

Two Schools of Treatment: Mutation Mimicry and Gene Excision

In the mid-1990s[7] it was found that a 32 base pair deletion in the CCR5 coding gene conveyed significant resistance to HIV through an inhibition of viral entry; it was shown a decade later in the case of the "Berlin Patient" (Timothy Brown) that the replacement of patient bone marrow with CCR5Δ32 donor marrow could effectively cure the patient of HIV.[8] This discovery has driven researchers to search for a way to induce or mimic the CCR5Δ32 mutation autologously, and solutions have been found in the realm of genomic editing.



HIV Timeline: Progression and Escalation[9]

Following viral integration, HIV enters a latent phase in which integrated DNA lies dormant for roughly a decade, before its ultimate reemergence and subsequent decimation of the immune system (AIDS). In the last two years, research has emerged

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[6] Atze T. Das, Alex Harwig, and Ben Berkhout, "The HIV-1 Tat Protein Has a Versatile Role in Activating Viral Transcription □," *Journal of Virology* 85, no. 18 (September 2011): 9506–16, doi:10.1128/JVI.00650-11.

[7] S. K. Cohn and L. T. Weaver, "The Black Death and AIDS: CCR5-Delta32 in Genetics and History," *QJM: Monthly Journal of the Association of Physicians* 99, no. 8 (August 2006): 497–503, doi:10.1093/qjmed/hcl076.

[8] http://defeathiv.org/berlin/

[9] https://en.wikipedia.org/wiki/HIV#mediaviewer/File:Hiv-timecourse_copy.svg

suggesting it might be possible to excise viral DNA during this latent period, and in doing so prevent escalation to AIDS.[10]

For the remainder of the paper, I will discuss in detail the three most well researched methods for the genetic treatment of HIV: Zinc Finger Nucleases (ZFNs; used primarily for CCR5Δ32 mimicry), TALENs (similar to ZFN in function and treatment), and the CRISPR-Cas9 system (gene excision).

Zinc Finger Nucleases

Zinc Finger Nucleases were the first serious tools for genome editing discovered, characterized in functional form in 1996.[11] They are hybrid enzymes, manufactured through the conjugation of the cleavage portion of bacterial Fok1 with synthetically engineered DNA-binding Zinc Finger enzymes. The Fok1 cleavage domain has no known sequence specificity, and so is capable of acting at any location on the genome;[12] Zinc Fingers are among the most well understood DNA-binding protein complexes, and in recent years it has become possible to select for complexes with specificity to a single genetic locus.[13] Working together, it is theoretically feasible to modify any location on the human genome with unprecedented accuracy.

A paper giving pre-clinical evidence for the safety of ZFN use in the inactivation of CCR5 was published in Nature last year,[14] and at present there are four registered

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[10] Wenhui Hu et al., "RNA-Directed Gene Editing Specifically Eradicates Latent and Prevents New HIV-1 Infection," *Proceedings of the National Academy of Sciences of the United States of America* 111, no. 31 (August 5, 2014): 11461–66, doi:10.1073/pnas.1405186111.

[11] Y. G. Kim, J. Cha, and S. Chandrasegaran, "Hybrid Restriction Enzymes: Zinc Finger Fusions to Fok I Cleavage Domain," *Proceedings of the National Academy of Sciences* 93, no. 3 (February 6, 1996): 1156–60.

[12] Dana Carroll, "Genome Engineering With Zinc-Finger Nucleases," *Genetics* 188, no. 4 (August 1, 2011): 773–82, doi:10.1534/genetics.111.131433.

[13] Martha L. Bulyk et al., "Exploring the DNA-Binding Specificities of Zinc Fingers with DNA Microarrays," *Proceedings of the National Academy of Sciences* 98, no. 13 (June 19, 2001): 7158–63, doi:10.1073/pnas.111163698.

[14] Lijing Li et al., "Genomic Editing of the HIV-1 Coreceptor CCR5 in Adult Hematopoietic Stem and Progenitor Cells Using Zinc Finger Nucleases," *Molecular Therapy* 21, no. 6 (June 2013): 1259–69, doi:10.1038/mt.2013.65.

clinical trials (three with clinicaltrials.gov,[15] and one with CIRM[16]) investigating its effectiveness in human subjects. Across all the studies, the treatment is the same: ZFNs specific for a region near the middle of CCR5 are introduced to isolated autologous hematopoietic stem cells. The gene is cleaved, severely disrupting the transcription of the CCR5 and effectively knocking it out. The modified cells are reintroduced to the patient, and cell count is monitored over some time span. The trial[17] and subsequent study[18] by Pablo Tebas et al. shows promising results; in particular, CCR5-modified cells exhibited four times the half-life of their unmodified cousins, and in one patient, HIV RNA became undetectable. The study's credibility is somewhat limited by its small cohort size (12, shrinking to 4 by the end of the study), and so the remaining clinical trials are attempting to remedy this by recruiting more participants (33 in Phase 1, at present[19]).

While ZFNs are promising, particularly in the presence of completed, positive clinical reports (none of the following techniques have entered clinical trials at the date of this report), they are somewhat limited in several important ways. First, they are difficult to manufacture, requiring a fairly involved synthetic modification of a highly conserved linker sequence in order to conjugate up to six individual fingers together (each finger conveys 3 bp of specificity). This process is lengthy, and expensive.[20] However, as CCR5 ZFNs have already been codified and are producible at scale, the time-delay in engineering can be ignored, leaving only cost as a consideration.

TALENs

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[15]http://clinicaltrials.gov/ct2/results?term=&recr=&rslt=&type=&cond=HIV&intr=Zinc+Finger+Nuclease&titles=&outc=&spons=&lead=&id=&state1=&cntry1=&state2=&cntry2=&state3=&cntry3=&locn=&gndr=&rcv_s=&rcv_e=&lup_s=&lup_e=

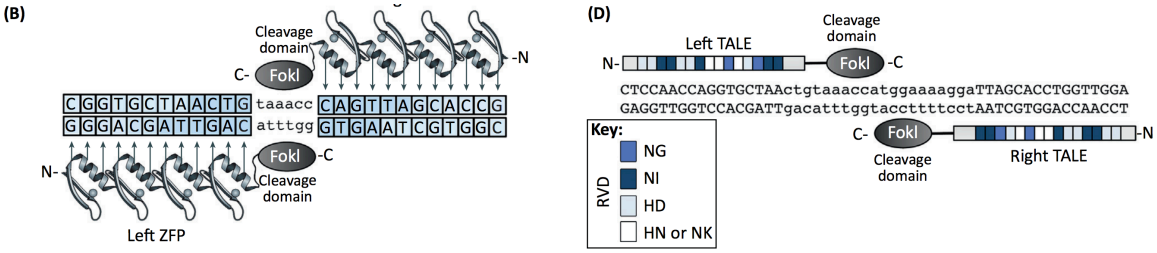
[16] http://www.cirm.ca.gov/our-progress/awards/ziinc-finger-nuclease-based-stem-cell-therapy-aids

[17]http://clinicaltrials.gov/ct2/show/NCT00842634?term=HIV+Zinc+Finger+Tebas&rank=1

[18] Pablo Tebas et al., "Gene Editing of CCR5 in Autologous CD4 T Cells of Persons Infected with HIV," *New England Journal of Medicine* 370, no. 10 (March 6, 2014): 901–10, doi:10.1056/NEJMoa1300662.

[19]http://clinicaltrials.gov/ct2/show/NCT01044654?cond=HIV&intr=Zinc+Finger+Nuclease&rank=3

[20] Thomas Gaj, Charles A. Gersbach, and Carlos F. Barbas, "ZFN, TALEN, and CRISPR/Cas-Based Methods for Genome Engineering," *Trends in Biotechnology* 31, no. 7 (July 2013): 397–405, doi:10.1016/j.tibtech.2013.04.004.



Comparison: ZFN (left), TALENs (right)[21]

Transcription activator-like effector nucleases, or TALENs, work in a manner similar to ZFNs but convey a greater degree of sequence specificity. TALEs (the effector without conjugated nuclease) are found naturally in *Xanthomonas* bacteria, and the discovery that they, too, are modular launched a highly successful field building on the preceding successes of ZFNs. Unlike ZFNs, conjugation of TALE subunits requires no reengineering, and following several injections of funding over the last couple of years, a library of TALENs has been assembled targeting 18,740 human genes.[22] Furthermore, higher base pair number specificity in TALENs means that constructs are likely to be more accurate in their action.

While no clinical studies exist, several *in vitro* studies have been conducted certifying TALENs as a viable alternative to ZFNs in genome editing.[23,24] Insofar as their application to the treatment of HIV, there can be no question that TALENs would be similarly efficient; however, due to the readily available supply of CCR5 ZFN constructs there is little supply-side incentive to replace ZFNs at the mode of treatment. This does not preclude a demand for clinical trials, however – as mentioned above, TALENs' heightened sequence specificity might convey some unforeseen advantage over ZFN-modified CD4+ T-cells. We will never know until we try!

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[21] Ibid., Fig. 1.
[22] Yongsub Kim et al., "A Library of TAL Effector Nucleases Spanning the Human Genome," *Nature Biotechnology* 31, no. 3 (March 2013): 251–58, doi:10.1038/nbt.2517.
[23] J. Keith Joung and Jeffry D. Sander, "TALENs: A Widely Applicable Technology for Targeted Genome Editing," *Nature Reviews Molecular Cell Biology* 14, no. 1 (January 2013): 49–55, doi:10.1038/nrm3486.
[24] Andrew J. Wood et al., "Targeted Genome Editing Across Species Using ZFNs and TALENs," *Science* 333, no. 6040 (July 15, 2011): 307–307, doi:10.1126/science.1207773.

CRISPR-Cas9

By far the newest addition to the genome editing class, CRISPR-Cas9 is a very powerful little piece of molecular wizardry. Derived from a prokaryotic cellular defense system against exogenous DNA,[25] CRISPR-Cas9 functions by inducing the expression of short RNA segments (induced in human cells via plasmid, lentiviral vector, injection, or some other means), termed single-guide RNA (sgRNA), which complex with the Cas9 endonuclease. This non-variable protein cleaves whatever DNA matches the genomic information on its sgRNA, thus silencing the target gene. The sgRNA, in turn, can be easily back-engineered from pathogenic DNA once the genome of the pathogen is known. The end result is an sgRNA:Cas9 nucleolytic complex with identical or superior specificity to ZFN or TALENs, that can be engineered on the genetic level without any engineering required on the protein level.[26] Given the great ease with which nucleic acid polymers like RNA and DNA can be manufactured (see introduction), and the myriad extant protocols for lentiviral plasmid expression in a cell, CRISPR-Cas9 has the potential to eclipse all other current genome editing technologies.

While CRISPR-Cas9 is a very new technology, several *in vitro* studies have been conducted confirming its efficacy against HIV infection. The specificity of the CRISPR-Cas9 system, combined with its ability to completely excise a gene,[27] makes it a fantastic candidate not only for CCR5 silencing, but for the total removal of latent HIV integrated DNA. A study conducted by Ebina et al.[28] showed that HIV expression-dormant T-cells, upon transfection with HIV LTR-targeting CRISPR-Cas9 genes, exhibited a significant loss (approximately 65%) of HIV genetic activity following reactivation. It was subsequently shown through sequence analysis that the CRISPR-Cas9 complex had in fact succeeded in cleaving and mutating the HIV genes.

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[25] Philippe Horvath and Rodolphe Barrangou, "CRISPR/Cas, the Immune System of Bacteria and Archaea," *Science* 327, no. 5962 (January 8, 2010): 167–70, doi:10.1126/science.1179555.

[26] Seung Woo Cho et al., "Targeted Genome Engineering in Human Cells with the Cas9 RNA-Guided Endonuclease," *Nature Biotechnology* 31, no. 3 (March 2013): 230–32, doi:10.1038/nbt.2507.

[27] http://zlab.mit.edu/assets/reprints/Ran_FA_Cell_2013.pdf

[28] Hirotaka Ebina et al., "Harnessing the CRISPR/Cas9 System to Disrupt Latent HIV-1 Provirus," *Scientific Reports* 3 (August 26, 2013), doi:10.1038/srep02510.

Recently, another treatment in the vein of CRISPR-Cas9 (technically speaking, RNAi [discussed here, omitted for interest of space] combined with an unrelated HIV fusion-inhibitor gene) has begun clinical recruitment.[29] Conducted by Calimmune, it combines an expression of short hairpin inhibiting RNA sequences specific to CCR5 mRNA with the expression of C46, a protein known to act as an antagonist to gp41 binding (gp41 being the other glycoprotein found on the HIV envelope).[30] I include this in the CRISPR-Cas9 section due to the similarity of the RNAi mechanism to that of CRISPR-Cas9; essentially, RNAi induces the detection and cleavage of unwanted mRNA, analogous to the sgRNA:Cas9-induced cleavage of genomic DNA.

Concluding Remarks

We are living in the age of the genome. As we continue to develop amazing new molecular machines for the modification of genetic material, we move further and further into the next stage of human development, a stage in which we have greater control than ever over our fates, our bodies, our dispositions, our very molecular makeup. Ethical implications aside, the sheer transcendent power of this next-level control is exhilarating, and its potential for curing all variety of human ailment – and providing all manner of human enhancement, should science choose to go down that path – is historic on a universal scale. We must continue to step forward with caution, and the vast web of clinical safety trials (and general cautious nature of the scientific community in general) will guarantee this. As a future participant in this revolution, I am awestruck as the potential for discovery that awaits, just around the corner.

Let's get started.

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[29] http://clinicaltrials.gov/ct2/show/NCT01734850?cond=HIV&intr=stem+cell&rank=18

[30] Marc Egelhofer et al., "Inhibition of Human Immunodeficiency Virus Type 1 Entry in Cells Expressing gp41-Derived Peptides," *Journal of Virology* 78, no. 2 (January 2004): 568–75, doi:10.1128/JVI.78.2.568-575.2004.