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The Past, Present, and Future of Gene Therapy

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ABSTRACT

Since 2010, gene therapy has rapidly gained interest as a possible method to cure previously untouchable and incurable diseases. The idea of tackling the disease at its genetic core to prevent the malignance from manifesting in the first place seemed unrealistic at first, but decades of research have started to bear fruit and these untouchable diseases suddenly seem mortal. CRISPR-Cas9, Chimeric Antigen Receptor (CAR) T-cell therapy, and adeno-associated virus (AAV) therapy utilize different gene engineering techniques to nullify previously incurable diseases. This paper serves as a comprehensive review paper which analyzes the mechanism, advantages, and disadvantages of these three gene therapy techniques. This paper states the genetic scissor mechanism of the CRISPR-Cas9 complex, and its difference from its predecessors, the artificial tinkering of the CAR t-cell therapy method and its subsequent utilization of the host's immune system, and finally the transduction potential of the AAV gene therapy. Finally, this paper states the current status and clinical application of the three gene therapy techniques, including their medication terminology and target diseases, and helps elucidate the future and potential of these three gene therapy techniques.

1. Introduction to Gene Therapy

According to the United States Food and Drug Administration, gene therapy is a technique that modifies a person's genes to treat or cure disease (2018). Indeed, the general public has heard of the whispers of gene therapy since the turn of the millennia but only after two decades into the millenia has gene therapy been seen in hospitals and clinics. However, during the two decades during which genetic engineering-based therapy methods underwent countless trial and error to be as precise and benign as possible, unestablished rumors regarding gene therapy had seeped into society's everyday conversation.

One issue regarding gene therapy that must be addressed is the question " Why do we need gene therapy?" Gene therapy was proposed to address diseases that could not be treated with conventional medicine. Although humanity's medical knowledge and treatment procedures are advancing at unprecedented speeds, some diseases such as Alzheimers, cystic fibrosis, and muscular dystrophy, have frustrated all practitioners of medicine due to their lack of cure. However, of the three, cystic fibrosis has recently been tentatively proposed as the next target of Chimeric Antigen Receptor (CAR)-T cell therapy, a state-of-the-art integrating gene therapy method that had already produced positive results in leukemia and lymphoma (Cooney et al., 2018; Allan et al., 2021). We need gene therapy to treat diseases that are inherited or caused by genetic mutation. It could offer potential cures that traditional treatment could not solve.

The concept of gene therapy was first introduced in the 1960s only a few years after the structure of DNA was identified (Yourgenome, 2017). Later in 1973, the first genetic engineering technique was developed (Cohen et al., 1973). And the first time gene therapy was used was in a clinical trial in 1990, where a retrovirus-based vaccine was first utilized in two patients. In 1996 the first nuclease was proposed by a research group at Johns Hopkins University. The zinc finger nuclease (ZFN), which mainly features the zinc finger domain and the Fok1 endonuclease, was used to improve the specificity of DNA sequence targeting (Kim et al., 1996). However, with the founding of

such precise gene engineering techniques, a justified call for concern was raised, and the next years were mostly used on drawing out ethical applications to gene research.

Gene therapy would once again enter the mainstream in 2009. In 2009, a genetic eye disease, retinal dystrophy, was treated with adeno-associated virus (AAV) vector, and became the first gene therapy approved by FDA (Stieger et al., 2009). In 2010 the TAL-effector nucleases (TALEN) were developed, also containing Fok1, but could bind into pacific DNA sequences more easily than zinc finger nucleases (Christian. 2010). In a short 3 years since their presentation, the mechanisms of TALEN were being fine-tuned by other research groups and even the TAL effectors themselves were being thoroughly scrutinized to provide better insight into their eventual gene engineering application. (Li and Yang, 2013; Mak et al. 2013). and finally, in 2012, a novel method was proposed that was even more precise than both the zinc finger nuclease and the TALEN. The CRISPR/Cas9 system was developed, and quickly became one of the greatest customizable gene editing systems to grace the gene therapy sector. In 2017 *in vivo* gene therapy started to gain recognition and proved to be specialized for targeting specific organs. CAR-T cell therapy, a treatment that modifies T cells to recognize and attack cancer cells was also introduced in the same year.

This manuscript will take an in-depth look into three mainstream gene therapy techniques: CRISPR/Cas9, CAR-T cell therapy, and AAV. These gene therapy techniques will have their main mechanisms and limitations introduced and will be followed with possible future analyses regarding the gene therapy technique.

2.1 Gene Therapy in the Past

CRISPR-cas9 is currently proposed as the main gene therapy method by various research institutes throughout the globe (Uddin et al., 2020), but its research could not have been done without studying the ZFN and TALEN. ZFNs are short protein motifs which consist of a zinc finger made out of a single alpha helix and two or three beta-pleated sheets, a DNA-binding domain (DBD), and a FokI restriction endonuclease to cleave the DNA at its target (Urnov et al., 2010; Razin et al., 2012). The introduction of the ZFN was revolutionary at its time. DNA-binding proteins were thought to utilize a two-fold symmetry method to bind to the DNA's double helix, but Zinc proteins were capable of linearly linking themselves to specific nucleic acid sequences on the DNA (Klug, 2010). As can be guessed, the recognition of the DNA domain is the key component of DNA sequence targeting genetic engineering. A ZFN usually has three to six "fingers", and each finger is capable of recognizing 9 to 18 base pairs. As such, the ZFN may be considered too "blunt" compared to TALEN and CRISPR-Cas9 in its usage. To overcome this advantage, a significant amount of resources must be introduced to make sure the domain and other components function properly (Khan, 2019).

The second generation of genetic editing techniques was TALEN. While both ZFN and TALEN mostly follow the same general process of breaking the double strand for genetic editing, TALEN is both more precise and economical. TALEN also utilizes a DNA binding domain like the ZFN, and like the ZFN which uses the natural tendencies of the zinc finger to bind to a particular domain, the TALEN uses transcription activator-like (TAL) effector proteins for DNA sequence recognition. These TALE proteins can be up to 35 amino acids in length, and can be modified to recognize specific DNA sequences to bind to; this customization potential is TALEN's advantage over ZFN.

Table 1. Comparison of ZFN, TALEN, and CRISPR-Cas9

	ZFN	TALEN	CRISPR-Cas9
Origin	Zinc protein was identified in a African clawed frog, *Xenopus laevis*	Tal effectors found in Xanthomonas bacteria	CRISPR repeat found in genome of Escherichia coli
Mechanism	Protein-DNA interaction	Protein-DNA interaction	DNA-RNA interaction
Multiplex Detection	Difficult	Difficult	Greatly possible

2.2 CRISPR-Cas9

TALEN proved to be more specific compared to ZFN, but TALEN-based gene engineering techniques could only be used for simple mutations (Wang et al., 2013). Hence scientists strived to figure out a different mechanism which was more efficient. The answer was found inside CRISPR-Cas9. The precise mechanism of the CRISPR-cas9 may seem difficult to people who have encountered it for the first time, but it can be simplified into 4 simple steps. As such, it will be proper to first introduce the components of the CRISPR-Cas9 complex system. CRISPR stands for clustered regularly interspaced short palindromic repeat, and it is an antivirus immune defense mechanism found in prokaryotes. As can be seen from its full name, CRISPR contains several short repeated (palindromic) DNA sequences which are repeated. This composition allows the CRISPR to act as a sequence-specific immune system towards bacteriophages.

Cas on the other hand, is also an abbreviation, but unlike CRISPR, it is not so straightforward. Cas stands for CRISPR-associated proteins, and these Cas proteins are either DNA-unwinding helicases or DNA sequence-snipping nucleases. Cas9 is an RNA-guided nuclease that works with a DBD from the CRISPR to target and cut the DNA at a specific site (Li et al., 2023). The main difference between the CRISPR-Cas9 system is in the target sequence recognition methodology. While ZFN and TALEN utilize a DNA-protein interaction for target sequence recognition, the CRISPR-Cas9 system focuses on a DNA-RNA interaction. More importantly, compared to ZFN and TALEN, the CRISPR-Cas9 system had a substantially lesser degree of off-target complications.

The CRISPR-Cas9 system had first seen usage on a human patient in October 2016 (Cyranoski, 2016). Cells that have been genetically modified using CRISPR-Cas9 were injected into a patient who had an advanced form of lung cancer. The patient's own lymphocytes were removed and treated with a proper cell death mechanism to combat the cancer cells. An advanced version of this trial was conducted in 2020, with 22 patients, and showed generally favorable results with little off-target editing (Lu et al., 2020).

However, there was also an incident where unapproved usage of CRISPR-Cas9 was detected. In 2018, He Jiankui of the Southern University of Science and Technology in China stated he altered the embryos of seven couples by using CRISPR-Cas9 to modify white blood cells (Normile, 2018). Not only was the procedure conducted quite close to the introduction of the CRISPR-Cas9 to the science community, the embryos in question were healthy and did not require any modification in the first place. As stated in the beginning, genetic therapy is a topic that has to be approached cautiously. The actions of He Jiankui may have set back the application of CRISPR-Cas9's clinical trials by several years.

A problem with the CRISPR-Cas9 system lies in its possible inaccuracy during gene editing. Additionally, the artificial act of cutting double-stranded DNA strands may induce gradual genetic damage to the host body. Finally, although it has fewer errors compared to ZFN and TALEN, the CRISPR-Cas9 system is not perfect, and off-target mistakes may happen. Fortunately, while multiple research teams and companies were searching to reduce genetically engineered errors both *in vitro and in vivo*, CRISPR-Cas9 has entered a new age, where it is called CRISPR 3.0 or CRISPR-Act3.0. Dr. Yiping Qi has been researching methods to improve the efficiency of the CRISPR-Cas9 system

by modifying the RNA protein interaction. In his other work, CRISPR-Act2.0, which served as a stepping stone to this current iteration, he and his team were inspired by the RNA-protein interaction of the bacteriophage MS2 RNA stem loops. By changing the current iteration of the structural guide RNA to look like that of the MS2 RNA stem loop, his team was able to improve the RNA-protein interaction of the CRISPR-Cas9 by a factor of three to four (Malzahn et al., 2019).

In CRISPR-Act3.0, Dr. Yiping Qi's research group genetically engineered a tRNA-gR2.0 (single guide RNA 2.0) to activate multiple genes at the same time. This multiplex gene activation system boosts an activation rate which is greater than its competitors by a factor of 4 to 6. Although his research was done mostly on plants, the premise of his results indicates a feasible method by which multiple metabolic pathway editing may be possible (Pan et al., 2021).

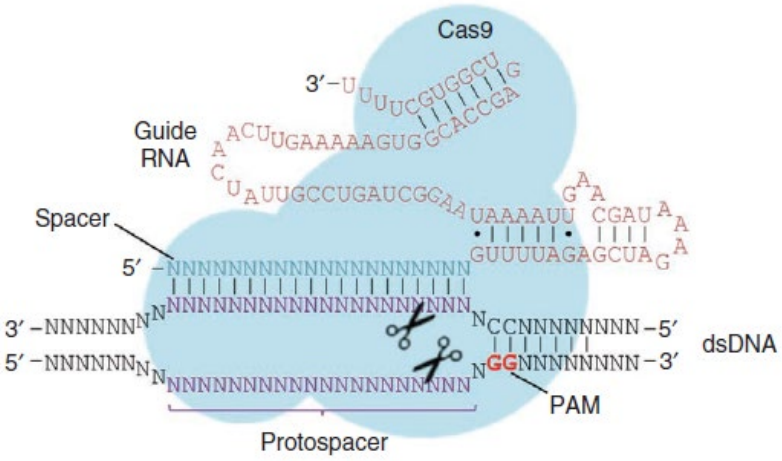

Figure 1. The Mechanism of the CRISPR-Cas9 system (Mali et al., 2013).

2.3 CAR-T Cell Therapy

Cancer treatment has traditionally relied on three major treatment methods: physical surgery, medicine-based chemotherapy, and energy-based radiation therapy. Recently, targeted therapy and immunotherapy, a treatment that enhances the immune system to target tumors, have emerged as prospective new treatment methods. CAR-T Cell therapy is one form of immunotherapy.

To properly understand the mechanism of Chimeric Antigen Receptor T-cell therapy, henceforth referred to as CAR-T cell therapy, an understanding of the CAR is required. The CAR is a recombinant receptor that is capable of influencing antigen binding and T-cell function (Sterner and Sterner, 2021). The CAR consists of four major components: the antigen-binding receptor domain, a hinge-binding domain, a transmembrane domain, and finally, a signaling domain.

To elaborate further, the antigen-binding receptor domain points toward the extracellular environment of the cell, and receives molecular signals which are specific to its receptor. As such, if the receptor itself can be tailor-made to a specific ligand, the specificity of the cell can be artificially increased, thus initiating an enhanced cell signaling cascade. The hinge bending domain and the transmembrane domain, which can be defined as a sequence of structural amino acids which support the domain region, the boundary region, and the transmembrane region (Zhang et al., 2013), will also have to be tailored to better support the artificial receptor with the equally important signaling domain. Finally, the signaling domain will also be genetically modified to induce a specific response. Additionally, more than one signaling domain may be attached to the CAR complex in order to bring about multiple intracellular signals.

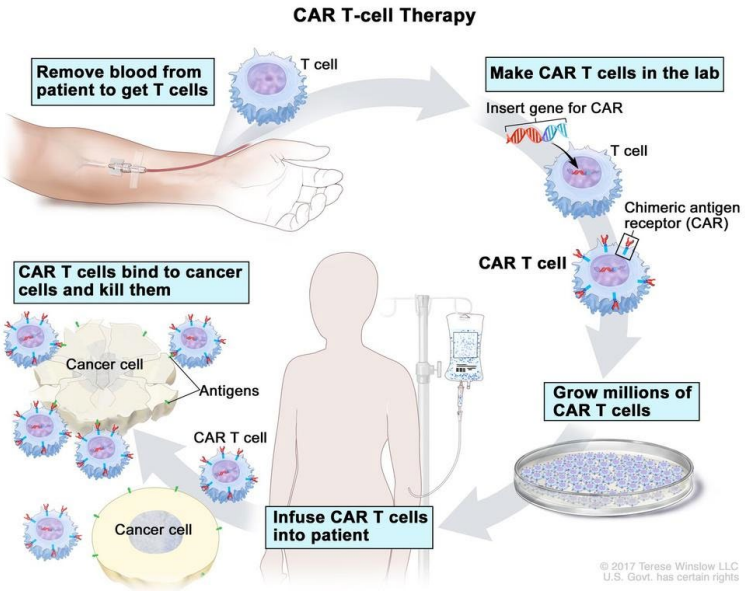

Figure 2. Extraction and modification of the CAR-T cell (NCI, 2023).

Specifically, the CAR is capable of repurposing an immune cell, such as the T cell, to locate a specific target antigen to eliminate. In essence, CARs can be thought to train a guard dog (T cell) to attack a specific type of intruder. Additionally, CARs have the ability to regulate the strength and frequency of T cells' response to an antigen (Sadelain et al., 2013).

As stated above the CAR-T cell is an artificial complex which utilizes the natural T-cells obtained from a patient. During the most recent decade, multiple variations of CAR-T cell therapy have been approved by the FDA for clinical use. OHSU Knight Cancer Institute has provided patients with KYMRIAH, an autologous T-cell immunotherapy for adults with follicular lymphoma (CBER, 2022; OSHU 2023).

Although not widely used, CAT-T Cell therapy showed the capacity to eliminate leukemias, lymphomas, and myeloma. However, it was also criticized for its costs (more than $450,000). CAR-T cells, in short, are customized by each patient based on the T-cells collected and are re-formed to produce CAR proteins, which will recognize and bind to the surface of cancer cells. With this new treatment, many patients were able to cure aggressive lymphomas, which seemed to be untreatable before the development of CAT-T cell treatment. However, there were cases where patients experienced critical side effects, such as dangerously low blood pressure, high fever, brain swelling, and seizures (NCI, 2023; OSHU, 2023). Additionally, acute toxicity side-effects such as cytokine release syndrome (CRS), hypoxemia, and hemodynamic instability were also recorded within 12 hours of infusion (AlDallal, 2020).

Yescarta is another FDA-approved drug that has been used in clinical environments since its approval in 2017. While the previously mentioned KYMRIAH targeted acute lymphoblastic, Yescarta, also known as axi-cel because of its usage of axicabtagene ciloleucel, focused on relapsed lymphoma (Lulla et al., 2018).

Axicabtagene ciloleucel targets cancer cells which exhibit CD19. CAR T-Cells which have been genetically manufactured as anti-CD19 CAR T-cells, target the cancer cells which express the CD19. As a result of this interaction, a chain reaction occurs where the ultimate fate of the cancer cell is cell apoptosis (Kite Pharma, 2020).

A clinical trial was held for Yescarta, and this contained 101 patients with diffuse large B-cell lymphoma, transformed follicular lymphoma, and primary mediastinal B-cell lymphoma, all of which can be classified as aggressive forms of lymphoma. For one month, these 101 patients were observed. An objective response rate was seen in 82% of the patients, and cytokine release syndrome was observed in 49% of the patients. Most importantly, however, were the results after six months. The six-month progression-free survival was observed to be 49%, and the frequency

of the disease reappearing after the six months was rare (AlDallal, 2020). However, toxic effects were also observed. The cytokine release syndrome was recorded to be the strongest within the first 12 hours of the CAR T-cell treatment, and neurological events were recorded to appear frequently for the first two weeks (Locke et al., 2017). Thankfully though, most of the patients recovered after the mentioned two weeks.

2.4 Adeno-associated viruses (AAV)

As mentioned above, the AAV was the first gene therapy method approved by the FDA in 2009 for a retinal-related disease. However, the history of the AAV goes back half a century. In 1965, scientists found fascinating biomolecules in the adenovirus (Atchison et al., 1965). These biomolecules would be coined adeno-associated viruses, and Atchison had identified three different archetypes of the AAV, and dubbed them AAV1 AAV,2, and AAV3. Upon further inspection of the AAV in the following years, the basic chemical structure of these AAVs was analyzed (Hoggan et al., 1966). Most noticeable was the discovery of a single-strand DNA genome inside the capsid of the AAV (Rose et al., 1969). However, its brilliance as a gene therapy vector was recognized when AAV was found to work together with the adenovirus for co-infection (Cheun et al., 1980). A decade later, the prototype of AAV gene therapy, known traditionally as recombinant AAV (rAAV), had a genetically modified AAV2 coding domain known as p*sub*201. This modified domain was a breakthrough in drug delivery as this rAAV not only had a 70% human cell infection rate but also was the first time AAV gene expression was not needed for gene therapy (Samulski et al., 1989; Hastie & Samulski, 2015). In general, the capsid of the AAV is the first indication of its penetration potential, and depending on the composition of the capsid structure, the subsequent reception by the target cell's receptors and endocytosis process may differ (Wu et al., 2006). Aside from the capsid composition, a variety of factors influence the AAVs gene therapy potential, one of which is the inverted terminal repeats (ITR).

By modifying the ITR of an rAAV, it is possible to increase the transduction potential of the rAAV. Because transcription and eventually, transduction, is hampered by the requirement of double-strand DNA (dsDNA), modifying the rAAV to bypass this step improves the transduction result. In this process, the promotor and transgene of the AAV are also modified to increase the speed of transcription and optimize transduction respectively (Li & Samulski, 2020). The best advantage of AAV is the ease with which the target DNA is injected into the host, hence its other name in vivo gene therapy. By utilizing virus-similar intrusion mechanisms, naked DNA is able to be inserted into the host. By tweaking the virus vector to not replicate itself, genetic engineers have produced a one-way transport mechanism for precise gene expression (Kotterman et al., 2015; Lundstrom, 2018). The adenovirus was one of many virus vectors that were chosen for the reasons above.

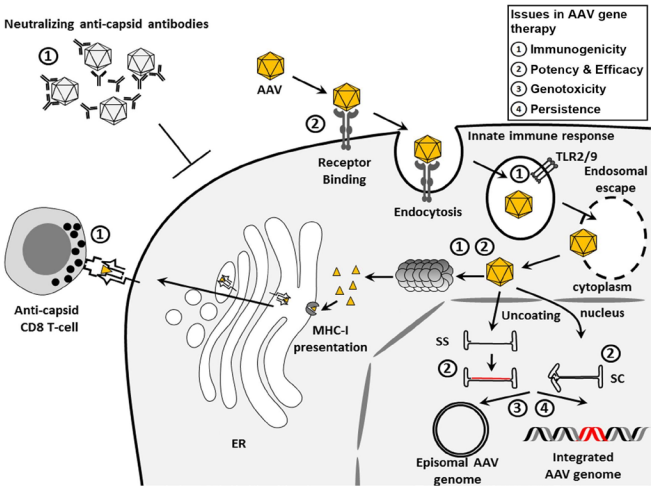

Figure 3. AAV mediated gene therapy (Colella et al., 2018)

Compared to CRISPR-Cas9 and CAR T-cell therapy, AAV gene therapy had been under the microscope of the gene engineering community for a long time. As such, its clinical applications had more time to be thoroughly inspected, and AAV gene therapy was able to gain the approval of the FDA at an early date. In 2012, a lipoprotein lipase deficiency medication known as Glybera was granted approval by the FDA (Büning, 2013). 4 years after that, another gene therapy medication that targeted a different target in patients with severe deficiency, Strimvelis, was also granted approval (Aiut et al., 2017). Once again, when compared to CRISPR-Cas9 and CAR T-cell therapy, AAV gene therapy is relatively easy to produce and therefore economic, has low immunogenicity, and most importantly, is non-pathogenic and thus minimizes harmful interaction with the host while maximizing gene expression (Hastie & Samulski, 2015).

As it stands, AAV is being assessed in a multitude of clinical trials. It has been assessed in inherited retinal and optic nerve degenerations (IRD), Parkinson's disease, and cardiac diseases (Moore et al., 2017; Deverman et al., 2018; Ishikawa et al., 2018). However, although the various clinical trials admitted positive results, it seems the natural immune system itself was a major barrier to the AAV. As such, modifying the AAV to induce minimal immune response while retaining its transduction rate is the main hurdle for efficient AAV gene therapy (Sack & Herzog, 2009).

3. The Future of Gene Therapy

The most recent decade has ushered in a turning point in gene therapy. Theoretical ideas have materialized in hospitals and medical institutions under the careful care of brilliant scientists and doctors. Although CRISPR-Cas9 cell therapy currently possesses the spotlight for gene therapy, other methods such as oligonucleotide-based therapies (Spinraza, Exondys 51, Vyondys 53), Chimeric antigen receptor (CAR)-T cell therapies (KYMRIAH, Yescarta, Tescartus), and AAV *in vivo* gene therapies (Luxturna and Zolgensma) have been approved for specialized usage. For example, spinal muscular atrophy, once thought to have been a disease which slowly consumes the locomotion mechanism of a human being, has been stopped in its tracks by an in vivo adeno-associated virus (AAV) gene transfer treatment method. Additionally, gene therapy techniques that have been already approved, have continued to undergo additional clinical trials to eliminate or minimize their size effects.

For example, an additional trial for Yescarta, aptly named a phase two ZUMA-12 trial, the previously mentioned anti-CD19 CAR T-cell therapy was applied again to 40 patients. However, unlike the ZUMA-1 trial which was applied to patients who have already undergone two cycles of therapy, the ZUMA-12 trial was a multicenter single-arm trial during the patients' two cycles of chemotherapy. The results were favorable and proved that gene therapy can be applied alongside the usual trifecta of physical surgery, chemotherapy, and radiation therapy (Neelapu et al., 2022).

In the case of AAV, additional virus vectors have been identified besides the adenovirus. Alphaviruses, measles virus, poxviruses, and picornaviruses are just a few of the possible virus vectors at genetic engineering's disposal (Kotterman, 2015). Additionally, like Yescarta, the AAV gene therapies are under constant scrutiny from the scientific community (Ogbonmide, 2023). In the case of Zolgensma, it was found to be tailor-made for treating type 1 Spinal Muscular Atrophy, and that it was particularly efficient in pediatric patients.

Although this manuscript has divided gene therapy methods into three main categories, it was done to introduce and help the reader understand the three major gene therapy contemporary trends. In the clinical field, these three gene therapy techniques work together to bring about their best results. The CRISPR-Cas9 treatment in 2020 for the 22 lung cancer patients was a joint effort between CRISPR-Cas9 and CAR T-cell. CRISPR-Cas9 was utilized to produce the artificial gene which was placed inside lymphocyte T-cells (Lu et al., 2020). Kymriah and Yescarta, although they were introduced under CAR T-cells, were produced using the lentiviral vector and retroviral vector respectively (Dunbar et al., 2018; Maude et al., 2018). Gene therapy's foreseeable future will include a crisscross of gene therapy techniques which serve to cover one another's fallacies.

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