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GENE THERAPY for the TREATMENT of X-LINKED RETINITIS PIGMENTOSA: A REVIEW

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Abstract: Gene therapy is a medical procedure that inserts a normal sequence of genetic material into living cells to rectify a malfunctioning DNA by replacing, altering or augmenting the defective form of the DNA. Gene Therapy has been known to be a promising treatment for genetic disorders, especially monogenic diseases such as Inherited Retinal Disorders (IRD). One of the most common types of IRD is X-Linked Retinitis Pigmentosa (XLRP), which is attributed to the mutation of the retinitis pigmentosa GTPase regulatore (RPGR) gene. Gene therapy using engineered CRISPR-Cas9 complex and Adeno-associated viral (AAV) vectors is capable to detect DNA mutation which could lead to an efficient transfection. This article reviews the underlying molecular mechanism of XLRP; the characteristics of the blood-retinal barrier (BRB) and other cellular structures of the retina as the ideal site to perform gene delivery; as well as recommended approaches in performing subretinal injection to achieve optimal outcomes.

Keywords: Gene therapy, x-linked retinitis pigmentosa, retinitis pigmentosa GTPase regulator (RPGR), adeno-associated virus (AAV)

INTRODUCTION

Over the last four decades, gene therapy has served a wide range of promises that are important in preventing morbidities and expanding life expectancy. Gene therapy can be defined as a medical treatment that inserts a normal sequence of genetic material directly into living cells to rectify a missing or a malfunctioning DNA through gene replacement, alteration or augmentation (Ong *et al.*, 2019). Long before its introduction to human embryo cells, gene therapy has been widely applied to conduct experiments on animal cells *in vitro*. Since the publication of a controversial experiment on genetic babies led by a Chinese biophysics researcher, He Jiankui, a number of ethical issues have emerged regarding its legality and thus has led to debates among ethicists and genetic scientists. At the same time, however, it has also opened the gate of hope to many people from all over the globe who has been suffering from non-curable diseases, especially genetic disorders. This genetic condition affects approximately 350 millions of people worldwide despite the rarity (Babar, 2017). One of the most common forms of genetic disorders, contributing to ten thousand types of human diseases is monogenic diseases (WHO, 1996). These diseases are caused by a single mutation in a single gene, which can be passed from parent to their child, causing inherited genetic diseases, such as Retinitis Pigmentosa (RP). RP is a group of Inherited Retinal Disorders (IRD), affecting 1 in every 4000 people worldwide, and is often described as a progressive genetic disease that attacks the photoreceptor cells, leading to irreversible

blindness (Dias *et al.*, 2018). One of the most severe types of RP affecting males in particular and accounts for 10 – 20% of total RP cases globally, is X-Linked Retinitis Pigmentosa (XLRP) (Kapetanovic *et al.*, 2019). The underlying molecular mechanism of XLRP is attributed to the mutation of the Retinitis Pigmentosa GTPase Regulator (RPGR) gene resulting in a fatal impairment of the expressed proteins leading to photoreceptor degeneration (Martinez-Fernandez De La Camara *et al.*, 2018). The complex pathophysiology of XLRP also gives rise to several challenging treatments. Several therapeutic agents such as neurotrophic drugs, anti-apoptotic agents and vitamin supplementations have shown to delay the progression of the disease (Hamel, 2006). However, they do not solve the leading cause of XLRP. Consequently, gene therapy emerged as a promising strategy to overcome the fundamental cause of the disease by introducing a precise gene into the retinal cells utilising viral vectors. One of the most well-defined viral vectors used in ocular gene delivery is Adeno- associated virus (AAV) (Wang, Tai and Gao, 2019). This review will consider a number of arguments informing the benefits of gene therapy; perspectives of different gene-editing tools and viral vectors; the perfect site and technique to perform gene therapy; and the future possibilities in gene therapy. Finally, it will argue that the best approach to treat X-Linked Retinitis Pigmentosa is genetherapy.

THE RETINA AND PHOTOTRANSDUCTION

The eye is a spherical and bilateral organ situated in the orbital cavity of the skull and is home to specialised structures responsible for vision. The eye is made up of three concentric layers or tunics: (1) the fibrous tunic which consists of the sclera and the cornea; (2) the vascular tunic which consists of the choroid, ciliary bodies and iris; and (3) the nervous tunic which consists of the vitreous body and the retina (Dias *et al.*, 2018). The eye can also be classified into two segments – anterior and posterior segment – with the lens being the border that separates the two chambers (Nguyen *et al.*, 2020). The most affected structures in hereditary retinal disorders, especially XLRP, are the photoreceptors of the retina. The retina is the sensory layer that sits between the choroid anteriorly and the vitreous body posteriorly. It is arranged in three main layers, including the photoreceptor layer, Browns Layer which comprises of bipolar cells, and the Retinal Ganglion Cell (RGC) layer (Megaw, Soares and Wright, 2015). Photoreceptors are cells that detect light and convert them into electrical impulses through a series of mechanisms involving proteins, enzymes and neurotransmitters, known as the phototransduction cascade (Dias *et al.*, 2018). These cells are the rods and cones, stimulated by dim and bright light, respectively. Both rods and cones are formed by three segments: (1) the outer segment (OS); (2) connecting cilium (CC); and (3) the inner segment (IS) (Berger, Kloeckener-Gruissem and Neidhardt, 2010). The OS is composed of flattened discs containing opsin pigment, retinal and transducin protein as well as phosphodiesterase (PDE) enzyme. The CC - a cylindrical organelle that connects the OS and IS together – is doing more than simply relaying the chemical signals to and from the segments (Martinez-Fernandez De La Camara *et al.*, 2018). It houses the responsible gene that becomes defected in XLRP, known as the Retinitis Pigmentosa GTPase Regulator (RPGR) gene (Megaw, Soares and Wright, 2015). Each segment carries its own specific genes to produce proteins in order to survive and maintain their role during phototransduction.

DISEASE MECHANISM OF X-LINKED RETINITIS PIGMENTOSA

The RPGR gene is located in the chromosomal region Xp21.1 of the X-chromosome arm. This gene possesses two main isoforms that are primarily concentrated in the Connecting Cilia, named RPGREx1-19 and RPGRORF15 (Lyraki, Megaw and Hurd, 2016). Both of

these gene variants are believed to encode proteins that will give rise to the non-motile cilia – an important structure that connects the outer and inner segments of photoreceptors. The interconnection between the segments organises the bidirectional pathway of proteins that are critical in the overall cell survival and viability. Mutations of the RPGR gene occur exclusively in the rods and lead to a malfunctioned connecting cilia, resulting in rod cell death (Deng *et al.*, 2015). Given that the rods are sensitive to dim light and scattered along the peripheral regions of the retina, their progressive loss will present aninitial symptom of night blindness (Dias *et al.*, 2018). This early clinical manifestation is often neglected because the cones are still able to compensate for bright vision. However, in most cases of XLRP, the secondary deaths of cones will eventually develop as time moves forward. A number of reported studies have introduced the mechanism of cone deterioration in XLRP. The first theory suggested that cones were highly dependent on a particular neurotrophic factor secreted by the rods, the Rod-derived Cone Viability Factor (RdCVF), which aimed to promote cell growth and maturity (Aït-Ali *et al.*, 2015). Another theory stated that cone degeneration aroused due to intracellular nutrient drawback. The excessive loss of RdCVFs also impaired the cellular binding of Basigin-1 (BSG-to cones leading to decreased glucose uptake (Narayan *et al.*, 2016). Other concepts such as microglial activation and oxidative stress have also been considered in supporting the cone cell deaths. If left untreated, this burden could further progress, leading to irreversible blindness.

ADVANTAGES OF GENE THERAPY

It is believed that Inherited Retinal Disorders do not require gene therapy for comprehensive treatment. The treatments of these genetic disorders are better performed using systemic pharmacological agents (Hamel, 2006). Firstly, neuroprotective agents such as neurotrophic factors and anti-apoptotic agents have been shown to spare photoreceptors' life cycle by promoting cell maturity and inhibiting programmed cell death, respectively (Wubben, Zacks and Besirli, 2019). In addition, alternatives such as Carbonic Anhydrase Inhibitors (CAI) and Anti-Vascular Endothelial Growth Factor (VEGF) agents were also prioritised due to their anti- inflammatory abilities to reabsorb fluid, stabilise electrolytes and prevent the formation of new leaky blood vessels (Schoenberger and Kim, 2013). However, gene therapy has been exposed as a promising treatment since it surpasses previous ocular medications that were believed to only delay the progression of the disease. Gene therapy provides a number of health benefits to treat IRDs. In fact, it is a well-known medical procedure that solves the underlying cause of diseases by correcting the genetic mutation situated in the specific loci of the cell DNA Jackson *et al.*, 2018). Furthermore, gene therapy provides at least three molecular strategies to enhance the immunogenicity of the mutated cells to stimulate immunological and physiological recognition. These strategies can be obtained by: (1) utilising rare-cutting endonucleases to cut and modify the targeted genes using Zinc Finger Nucleases (ZFN) and Transcription Activator-Like Effector Nucleases (TALEN) (Lee, Kim and Hur, 2018), (2) relying on Homologous Direct Recombination (HDR) and Non-Homologous End Joining (NHEJ) effects to induce self-repair mechanism of the gene using CRISPR-Cas9 complex (Hung *et al.*, 2016); and (3) using Viral Vectors as gene hosts to enter the targeted cell using AAV Vectors (Li and Samulski, 2020).

GENE EDITINGTOOLS

The aim of targeted gene therapy is to enlist a DNA repair pathway by breaking down the intertwined double helix to form independent recombination with the insertion of the exogenous donor template (Hung *et al.*, 2016). Many scientists believe that Rare-Cutting Endonucleases (RCE) are the most ensuring gene-editing tool to achieve this final goal because they operate by manipulating endonuclease proteins to obtain Double Strand Breaks

(DSB) in longer gene sequences. Two of the most popular RCEs are ZFN and TALEN (Humbert, Davis and Maizels, 2012). ZFN is made up of DNA-binding domains fused with a *Fok-1* restriction enzyme and zinc fingers proteins that are able to cut a targeted sequence of DNA by recognising a cluster of 4 groups of 3 base pairs. Similarly, TALEN is mainly composed of identical domains except with TAL effector proteins that are capable of slicing a targeted DNA sequence by identifying a set of 33 repeats of an amino acid chain (Lee, Kim and Hur, 2018). However, the usage of endonucleases has been replaced by a more advanced genomic editing technology known as Clustered Regularly Interspaced Palindromic Repeats (CRISPR) (Adli, 2018). CRISPR sparked a revolution in the genetic field to overcome the complexity of protein cloning and engineering. In fact, CRISPR is distinguished by its simplicity since it is more efficient in adjusting single gene arrangements (Burnight*etal.*,2017). Therefore, the total length of the desired sequence that will be delivered can be decided independently in order to function correctly based on the molecular size of the gene and the features of the targeted cell. Moreover, CRISPR is more precise and flexible in establishing an exact cutting point of the DNA because it involves two essential components. The first essential component is the guide RNA (gRNA) that identifies and determines the cutting point of the DNA by the second component, which is the CRISPR- associated protein 9 (Cas9) (Adli, 2018). This protein complex cleaves the desired sequence escorted by the gRNA and allows promoting genetic modifications in the gap caused by the DSB. Together, they both form the CRISPR-Cas9 complex. Furthermore, the CRISPR-Cas9 complex enables to activate a self-repair mechanism subsequent to a DNA lesion via two types of mechanism (Burnight *et al.*, 2017). Firstly, an HDR can be achieved through a matched exogenous donor template with the site of the break, which results in gene correction. Alternatively, a second self-repair mechanism can be attained through small deletion mechanisms due to interaction between the lesion site and the template, which results in gene deletion (Lee, Kim and Hur, 2018). Once the engineered CRISPR-Cas9 complex is equipped with the gRNA, it is then ready to be delivered into the host cell utilising viral vectors. In order to achieve a successful gene delivery, genetic information requires the most suitable viral vector that could lead to an efficient transfection.

ADENO-ASSOCIATED VIRUSVECTORS

Viral Vectors have been known to be the most effective supporting vessel to accommodate engineered genomes for gene delivery into host cells compared to non-viral vectors (Naso *et al.*, 2017). Due to their unique and complex structures, most viral vectors are selective in binding to their receptors. They also rely on their limited content capacity in order to enable viral packaging modification before being transferred. On the basis of this theory, it is believed that AAV vectors are effective mediators to overcome monogenic diseases not only in animals but also in humans. Adeno-associated virus is a part of the Parvovirus family comprising two fundamental genes - *rep* and *cap* gene – flanked by Inverted Terminal Repeats (ITR) on each end of the single-stranded DNA (Ramlogan-Steel *et al.*, 2019). The ITRs are notably responsible for vector production, gene expression stimulation, and continuous cell transduction. The cap gene generates the structure of the capsid, while the rep gene supports the replication and aggregation of the AAV genome and virion, respectively. Leaving both ITRs intact enables approximately 96% of the AAV genome to be substituted with manipulated transgenes. In fact, replacing both cap and rep genes with a promoter and a donor template of normal transgenes contained in an expression cassette establishes the core function of AAV vectors (Ong *et al.*,2019).

To simply understand the vector transfection, the process undergoes a series of cellular actions. First of all, the AAV vector binds to the target cell membrane and attaches its virion

to receptors and co-receptors found in the surface of the cell. Then, the virion penetrates the cell and encounters the endosome through endocytosis. From this point, the virion is released from the endosome, transported to the nucleus, and disengaged from the AAV genome. Finally, the genome then is transformed from a single to a double-stranded DNA to undergo transcription and translation processes before eventually attaining the expression of the therapeutic transgene (Li and Samulski, 2020). Although the onset of gene expression carried by these vectors might be time-consuming, the prolonged sustainable outcomes of the expressed gene could last up to several years.

Another point to consider is that AAV vectors are non-pathogenic because they are equipped with non-enveloped capsid, which prevents causing local reactions in the surrounding tissue during or after the delivery (Naso *et al.*, 2017). These vectors also reduce the risk of insertional mutagenesis since they reject any form of integration or consolidation of large genes with long regulatory sequences. Moreover, they have the advantage to restrict any interpolations of unnecessary genes due to their small capacity (Wang, Tai and Gao, 2019). Therefore, it makes them transferable to particular tissues that are explicitly composed of non-dividing cells, which also supports the fact that the retina is an ideal site for the delivery because it is comprised of light-sensitive non-dividing nerve cells, which are known as photoreceptors.

THE RETINA: IDEAL SITE FOR GENETHERAPY

On the issue about the application of gene therapy in human cells, scientists have been considering the retina as the perfect site to perform the treatment. Due to its propitious anatomical, biochemical and immunological features, the eye, retina in particular, has been at the vanguard of gene therapy to treat IRDs (Dias *et al.*, 2018). The first rationale to support this statement is that the retina is home to photoreceptor cells – primarily rod cells – which are the main targets of the therapy. Rod cells are composed of three critical segments that are also influential in maintaining its life-cycle (Kapetanovic *et al.*, 2019). The first segment is the outer segment which is packed with a high concentration of proteins responsible for light detection and conversion, known as Rhodopsin. The second segment is the connecting cilium, a cylindrical structure containing transition fibres that connect and transmit the signal from the outer to the inner segment. The connecting cilium is the site where the RPGR gene mutation occurs, resulting in the termination of the proteins responsible for signal transmission. The last section is the inner segment, a relay station that forwards the electrical signal to the optical tract via the optic nerve before being processed in the primary visual cortex in the occipital lobe of the brain (Berger, Kloeckener-Gruissem and Neidhardt,2010).

The second reason that makes the retina suitable for the procedure is due to its privileged immunological structure known as the Blood Retinal Barrier (BRB). The BRB is a structure separating the neuronal retina from the fragile choriocapillaris – a consolidation of arteries and venules supplying the retina that are originated in the choroid (Dias *et al.*, 2018). The strategic location of the BRB encompasses at least three fundamental roles. Firstly, it maintains the physiological environment by balancing ion concentrations to ensure the viability of the entire retinal structure. Secondly, the barrier limits the immunological reaction locally without involving systemic inflammation spread due to the absence of lymphatic vessels. Indeed, the barrier would rather trigger phagocytosis, an engulfing process of particles by specialised cells, particularly macrophages in the retinal layer. Lastly, the BRB participates in the removal of cellular debris and the apoptotic photoreceptors (Nguyen *et al.*, 2020). Given that the retina is one of the most active tissues metabolically

and owing to the phototransduction cascade borne by the photoreceptors, continuous photon exposure will also generate large quantities of metabolic waste. However, in XLRP, considering that the photoreceptors carry mutated genes, programmed cell death develops due to malfunction characteristics of the genetic material instead of its apoptotic nature. In the end, after the insertion of the constructed genes to the retina, purines and pyrimidines - both known as biochemical compounds composed of nitrogen and carbon chain that are involved in DNA and RNA formation - will accumulate as end- products resulting from transcription and translation processes (Lyraki, Megaw and Hurd, 2016). For this reason, the BRB will be responsible for the transport of these metabolic wastes from the retina via the choriocapillaris.

The final related argument that bolsters the previous statement is that the retina is accessible to sophisticated diagnostic tools and treatments. The retina enables the examination using advanced modalities to confirm or eliminate other possible diagnoses (Hamel, 2006). This can be done by utilising internal imaging of the eye. General findings such as precise ocular illustrations and qualitative measurements of the inner structure can be obtained with the aid of Optical Coherence Tomography (OCT), Confocal Scanning Laser Ophthalmoscopy (CSLO), or Scanning Laser Polarimetry (SLP) (Chadderton *et al.*, 2009). Ocular imaging also provides a specific examination of the retina with the assistance of (1) Microperimetry, which determines the defect of visual acuity and retinal sensitivity; (2) Fluorescein Angiogram, which helps to visualise the blood flow in the retina; and (3) Retinal Tomography, which displays the detailed structure of the retinal layers (Hamel, 2006). Aside from diagnostic concerns, the retina is also assessabletoperformminimally-invasiveproceduresinordertoensuretheviralvectorsafelyreaches the target cell during the delivery. Such procedures can be executed through intravitreal or subretinal injection.

SUBRETINAL INJECTION

The intravitreal injection has been used dominantly to treat vitreoretinal diseases because it is less invasive and performs directly to the anterior segments of the eyeball (Deng *et al.*, 2015). However, due to the extensive range of the injection method, a number of complications and side effects have been reported after the procedure, such as bleeding, retinal holes, glaucoma, or even worst, endophthalmitis. As a result, these complications can cause increased intraocular pressure, leading to legal blindness (Kapetanovic *et al.*, 2019). This unsatisfactory method has led scientists to develop a novel approach to manage and examine the imperceptible hind area of the eye. The subretinal injection was then introduced as a promising technique to deliver the vector in XLRP patients. The subretinal injection is able to pass through the prominent barriers, such as the vitreous and the inner nuclear membrane of the retina, which are known to be impenetrable by AAV vectors (Hartman and Kompella, 2018). Thus, this technique holds the possibility to perform directly to the posterior part of the retina by detaching the photoreceptor layer from the RPE. The detachment is facilitated by the subretinal bleb during the injection, allowing the therapeutic agent to bind and diffuse to the photoreceptors (Nguyen *et al.*, 2020). In addition, this method is more advantageous compared to intravitreal injections in terms of entry points since it allows the operator to enter the retina via three different routes. Due to the flexibility, the injection can be carried out through a transcorneal and transscleral approach, both known to reach the subretinal space by penetrating the retina through the pupil and limbus areas, respectively. It is also possible to reach the subretinal space by penetrating the choroid without piercing the retinal layer through a slight modification of the transscleral route (Hartman and Kompella,2018).

The results of ocular gene therapy using the subretinal injection have reported successful outcomes, both from animal and human trials. A study using the subretinal injection to compare the treated and untreated retinas conducted in a group of XLRP mice reported three appealing results: (1) using a light microscope, the treated retinas showed three additional dense rows of cells lining up in a neat arrangement; (2) using an electron microscope, the treated retinas maintained the structures of the outer segment discs; and (3) using immunofluorescence staining, the treated retinas showed obviously visualised OS and IS with highly distributed Rhodopsins, all measured at three months post-delivery compared to the untreated eyes (Pawlyk *et al.*, 2016). A similar study also reported significant preservation of the rods and cones at 2, 3 and 5 months after the delivery compared to the controlled retinas using Photoreceptor Electroretinogram (Chadderton *et al.*, 2009). Furthermore, a current cohort study conducted in human retinas showed a progressive improvement in retinal sensitivity and visual acuity from the initial to 6 months post-therapy compared to the controlled retinas using microperimetry and EDRS Charts (Cehajic-Kapetanovic *et al.*, 2020).

FUTURE POSSIBILITIES FOR GENETHERAPY

In 2018, the first FDA-approved ocular gene therapy, *Voretigene Neparvovec* (Luxturna®), was used to treat an IRD caused by biallelic mutations of the RPE65 gene, leading to a disease known as Leber's Congenital Amaurosis (LCA) (Ong *et al.*, 2019). Today, a number of phase 1 and 2 trials have been conducted to understand the underlying molecular mechanism as well as to prove the safety and efficacy of gene therapy to treat XLRP. At this rate, the continuation of the trial should move forward to a larger sample size of randomised clinical trials in order to achieve the approval of gene therapy as the standard treatment to cure XLRP. There is no doubt that gene therapy will play a significant role in future science. Current gene therapy trials have shown the improvement of statistically significant survival rate in hereditary disorders and may be possible to be used to cure other deadly diseases, such as cancer, immunodeficiency diseases, as well as neurodegenerative diseases (Cross and Burmester, 2006). With the rapid development of gene editing modalities, gene therapy can lead future biomedical researches to investigate chromosome manipulation and epigenetic regulation which are believed to be far beyond genome editing (Adli, 2018).

CONCLUSION

In conclusion, the implementation of gene therapy on XLRP patients is becoming rapidly mature, and has been widely accepted ethically on human experiments. As mentioned above, this essay has discussed the features of gene therapy in overcoming the complexion of XLRP. As retinal genetic disorders may become a major threat to those who are at high risk, the community, especially men with familial history of inheritance, should no longer delay their physical and genetic examinations since acquiring an early treatment is as important as undergoing an early detection of mutation. In addition, the effectiveness of these gene-editing treatments can offer the potential for promising outcome to treat non-curable diseases and can also exist as the most favourable approach in terms of personalised medicine. In the future, there will be great hope that the invention of these modern medical technologies will support the shifting of XLRP to a manageable, progressive disease without prolonged suffering and morbidity.

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