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Advancements in Non Viral Gene Therapy for Hemophilia

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Abstract

Over the last 10-15 years, significant advances in vector design and delivery techniques have facilitated the development of nonviral approaches for the treatment of hemophilia. Despite these advancements, there remain several obstacles preventing the successful application of these approaches in larger mammals such as dogs and humans. This review covers nonviral gene therapy approaches using both *in vivo* gene delivery and *ex vivo* gene transfer. Plasmid-based approaches, as well as integrating transposons are examined for efficacy, risks and limitations. Results are presented on the only human clinical trial in hemophilia that utilized nonviral approaches.

Keywords: Nonviral; Gene therapy; Transposon; Integrase; Hemophilia; Hydrodynamic

Introduction

Hemophilia is a disorder of the blood coagulation cascade caused by deficiencies in Factor VIII (FVIII, hemophilia A) or Factor IX (FIX, hemophilia B). Both diseases are X-linked and primarily affect males. Severe hemophilia (<1% activity of FVIII or FIX) results in recurrent musculoskeletal bleeding into joints and muscles causing pain and disfigurement. Current therapy for hemophilia involves intravenous infusion of recombinant coagulation factors which are effective at controlling bleeding, but are expensive and administered frequently (every 8-12 hours during active treatment). Hemophilia A and B are diseases particularly well suited for gene therapy strategies as small increases in circulating factor levels have significant clinical impact and benefit for the patient. There are a number of excellent reviews that cover current advances in the genetic treatment of hemophilia [1-3]. Many of these reviews emphasize viral methods of gene delivery, which are effective and currently being evaluated in clinical trials. Despite current clinical interest, viral methods have several limitations, including induction of the immune system. Nonviral gene delivery for hemophilia is an emerging field that has made steady progress toward clinical application. This review focuses on recent advancements in nonviral gene delivery and the obstacles that remain preventing clinical use.

Nonviral Gene Therapy – Advantages/Obstacles

Nonviral gene delivery approaches have several advantages over viral-based gene delivery. One major advantage is the reduced cost and simplified production of DNA compared to more complicated viral production. Purified DNA is generally stable, can be lyophilized for packaging and has a long-shelf life [4]. Nonviral gene delivery methods are less immunogenic as they contain no viral protein antigens that may activate the immune system. Activation of the immune system against viral epitopes present in viral vectors can lead to the destruction of virally transduced cells and loss of gene expression [5]. Furthermore, immune memory cells may block the ability to re-administer the viral vector once immunity develops. Lastly, most gamma retroviral-based vectors systems tend to integrate in or near actively transcribed genes leading to insertional mutagenesis. If the insertion event leads to proto-oncogene activation or disruption of a tumor suppressor gene, a malignancy could result as seen in several clinical trials [6]. Nonviral gene delivery methods are less likely to cause insertional mutagenesis providing increased safety; however this increase in safety is dependent on the type of vector used. Episomal vectors do not integrate and therefore should cause no mutations, while integrating nonviral approaches utilizing transposons or integrases still have the potential for insertional mutagenesis, but at a reduced frequency. Despite the potential advantages of nonviral delivery, a number of problems with nonviral vectors exist, including delivery issues and lack of persistent long-term expression. The immunological effects of different nonviral gene delivery methods and the development of inhibitory antibodies are beyond the scope of this review, yet are another important facet of gene therapy for hemophilia.

Nonviral Gene Delivery – Chemical and Physical Methods

The delivery of nucleic acids across biological membranes is challenging and generally requires chemical or physical methods to enhance delivery. There are several well written reviews that explore these topics in greater detail [7,8]. In most cases, the nucleic acids are in the form of circular plasmids, but linear DNA or RNA can be delivered. Situation specific goals will influence the type of nucleic acid utilized. Circular plasmids are commonly used because of their ease to generate in bacteria and the closed supercoiled nature of the DNA may provide some protection from degradation by serum endonucleases during delivery. Linear DNA, although more unstable, may be the preferred form of nucleic acid in certain situations as they can from concatemers [9]. RNA is appropriate if transient gene expression is desired, such as transposase expression. Regardless of the choice of nucleic acid, chemical or physical methods are required for efficient gene delivery.

Chemical methods

Chemical methods use compounds such as cationic liposomes or cationic polymers (such as polyethylenimine) to compact and package the nucleic acids into nanoparticles. Packaging of the nucleic acids helps to protect them from nucleases during *in vivo* delivery and facilitates passage of the genetic material inside cells and past endosomes to gain

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entry to the nucleus. Chemical methods work well for transfecting cells in culture, but when used *in vivo* can cause toxicities including aggregation of blood cells [10], activation of complement [11], cytokine activation and cytotoxicity to the tissue or organ exposed to the highest concentration of the complexes [12,13]. While these drawbacks have limited the use of these formulations in humans, chemical-based approaches to nonviral DNA delivery have been used in small animal models of hemophilia [14,15].

Systemic intravenous administration of chemical DNA complexes leads primarily to deposition within the first capillary bed the complexes encounter, mainly the lung, but also distribute to other organs including the liver, spleen, kidneys and heart [16]. Polyethylenimine is a linear or branched polymer that is a popular reagent to deliver DNA due to commercial availability, and improved efficacy [17]. Linear PEI (22 to 25 Kd) is more efficacious then cationic liposomal formulations and less toxic than branched forms of PEI [18,19]. However, PEI does have toxicity when used *in vivo* especially at higher concentrations, which limits use in larger animals and humans [20]. New biodegradable cationic polymers with less toxicity include aminoesters and chitosan, yet efficiency *in vivo* is not substantially different than linear PEI [21-23]. More complex chemical formulations exist where the DNA complexes are packaged into lipid-polymer hybrids, specialized nanoparticles or are engineered to have ligands for cell receptors on their surface, but are more difficult to produce [7]. A recent study by Kern et al., utilized a nanocapsule formulation designed to target hepatocytes and liver sinusoidal endothelial cells to treat hemophilia [15]. While these improvements have reduced toxicity, investigators are still generating new intravenous formulations with the hope of increased efficacy and applicability to human clinical trials.

Chemical formulations can be designed for alternative routes of administration including inhalation, topical and oral. While inhalation and topical delivery of DNA may not be relevant for the treatment of hemophilia, non-invasive oral approaches have merit [24]. Chitosan is a biodegradable cationic polysaccharide that forms complexes with DNA. Chitosan is thought to protect the DNA from digestion and increase the cellular uptake. Two groups have used chitosan/DNA nanoparticles to deliver the FVIII gene to cells within the gut [25,26] resulting in transient FVIII expression. The therapy was safe with repeated administration and there was no development of inhibitory antibodies.

Physical methods

Physical methods of nonviral DNA delivery involve a host of approaches that use physical energy to facilitate the passage of nucleic acids through biological membranes. The methods include pressure-based approaches such as hydrodynamic, direct injection methods, electroporation, ultrasound-enhanced and magnetofection. In many of these approaches toxicity is reduced since the nucleic acids are naked or non-formulated. Most of the adverse effects observed with these physical methods are due to the physical energy, which is self limited. Generally, the tissues/cells recover quickly allowing re-application of the approach.

In hydrodynamic delivery, naked DNA is administered intravenously via tail vein injection using a large volume (~8-10% of body weight) over a brief period of time (5 to 10 sec). The hydrodynamic method was developed in mice as the animals could survive the large change in blood volume by expanding their liver leading to deposition of DNA within hepatocytes [27,28]. Additional organs that receive DNA after hydrodynamic gene delivery in mice include the kidney, spleen, lung and heart. When standard plasmids are delivered, transient expression was observed in up to 30-40% of hepatocytes, however expression wanes over the next few days and then falls considerably (~10^3 fold loss) to low yet detectable levels by 1-2 weeks. Over the next 2-3 months expression usually becomes undetectable. This approach is routinely applied in small animal models of hemophilia for testing of transposon-based systems and other non-integrating vectors.

Unfortunately, the hydrodynamic delivery approach is not easily adaptable to larger animals. Dogs and humans would not survive the large volume injections required to swell the liver. Mechanical approaches involving intravascular catheters with balloons to isolate vasculature within specific lobes of the liver were first developed in rabbits [29], and subsequently tested in larger animals such as swine [30-32]. These successes led to a small trial in humans delivering the thrombopoietin gene [33]. Attempts at treating hemophilic dogs using catheter-mediated hydrodynamic therapy are underway at the University of Minnesota under the direction of Drs Hackett and McIvor. Their recent article suggests that these approaches still need optimization before they can be considered in humans [34].

Obtaining long-term gene expression

While chemical and physical methods of DNA delivery are moderately efficient at delivering nucleic acids to cells and tissues, a second major obstacle to effective nonviral delivery is the lack of long term-expression. The reasons for transient gene expression are complex, and include a combination of cellular activities including the degradation or extruding of non-integrated plasmid DNA, transcriptional silencing of the exogenous plasmid or apoptotic cell death. Over the past decade, investigators have been modifying vector designs in order to facilitate long-term gene expression and significant advances have been made.

Transposons and integrases

One of the major advances in promoting long-term gene expression in nonviral systems has been the use of transposons and integrases to facilitate genomic integration of the delivered genes. The first use of transposons to treat hemophilia came from the laboratory of Mark Kay where the *Sleeping Beauty* transposon system, originally characterized by Ivics [35], was used in combination with hydrodynamic gene delivery to treat adult hemophilia B mice [36]. The results showed that with a mutant transposase, FIX expression was only transient with undetectable FIX levels by 60 days. With the transposase, FIX expression stabilized at ~100 to 200 ng/ml and continued until the end of the experiment (5 months). The same approach was used to treat adult hemophilia A mice; however, these mice developed inhibitory antibodies against the gene product, FVIII, limiting expression was unless the mice were tolerized prior to gene delivery [37]. Similarly, our own laboratory used the *Sleeping Beauty* transposon to deliver the FVIII gene to neonatal mice using a chemical approach (PEI) [14]. Because the immune system of neonatal mice is naive, these mice did not develop inhibitors and thus, did not require tolerization. Other transposons such as the piggyBac transposon have been used to facilitate long-term expression within the liver [38], but have not been used for the treatment of hemophilia to date.

Since the initial discovery of the *Sleeping Beauty* transposon system, improvements have been made to the system to facilitate efficient integration. Significant improvements came from the development of hyperactive transposases with increased activity [39,40]. Transposon vectors may also incorporate other cis-acting regulatory elements that

enhance integration [41]. Finally, transposon systems can be combined with viral vectors to prolong the duration of gene expression as in this example using a high capacity adenoviral vector to delivery FIX to dogs [42].

While transposon systems clearly enhance expression over time, the fact that transposons integrate increases the risk of insertional mutagenesis. Several studies investigated where transposons integrate within the genome [43-45]. The results of these studies suggest that both the *Sleeping Beauty* and piggyBac transposases have slight biases towards integration near transcriptional start sites and microsatellite repeats. Despite these biases, they appear safer than retroviral vectors, which have an increased tendency to integrate near actively transcribed genes. Therefore, there is inherent risk of insertional mutagenesis using any integrating system including transposon-based approaches.

Our laboratory and others have attempted to minimize the risks associated with transposon insertional mutagenesis by forcing the *Sleeping Beauty* or piggyBac transposons to integrate at specific sites within the genome [46-48]. While some advances were made on this front, these site-specific transposases still have a significant fraction of non-targeted integration events that may cause insertional mutagenesis. Lastly, there is a concern that if the transposase gene is expressed over time in cells that have a transposon there may be multiple excision and integration events that would further increase the risk of insertional mutagenesis. A recent study examined the persistence of the plasmid expressing the transposase gene in T cells [49]. Although there was no detectable protein by Western blot, PCR analysis was positive in 5 of 15 bulk T cell populations that underwent nucleofection and selection for the transposon gene [49]. Due to these risks, one group has proposed using RNA as the source of the transposase gene, which cannot integrate and thus, cannot lead to long-term transposase expression [50].

Another approach to facilitate long-term expression is to use integrases that integrate in a more site-selective manner. A prime example is the phiC31integrase pioneered by Michelle Calos [51]. In this system, the phiC31 integrase mediates the integration of plasmids bearing an *attB* site into endogenous sequences in the genome with partial identity to the phage *attP* site (pseudo-*attP* sites). A comparison of the mechanisms of integration of the *Sleeping Beauty* transposon and the phiC31 integrase is presented in (Figure 1). Based on analysis of integrations within the human genome, there appears to be a limited number of pseudo-*attP* sites that are targets for integration with several sites seemingly preferred over others. For example, a region on chromosome 19q13.31 was targeted 7.5% of the time [52]. The limited number of potential integration sites should reduce the risks of insertional mutagenesis, yet this hypothesis has not been tested. The phiC31 integrase has been used to correct hemophilia B in murine systems [53,54]. Finally as with the *Sleeping Beauty* transposase, there is concern that persistent low level expression of the phiC31 integrase

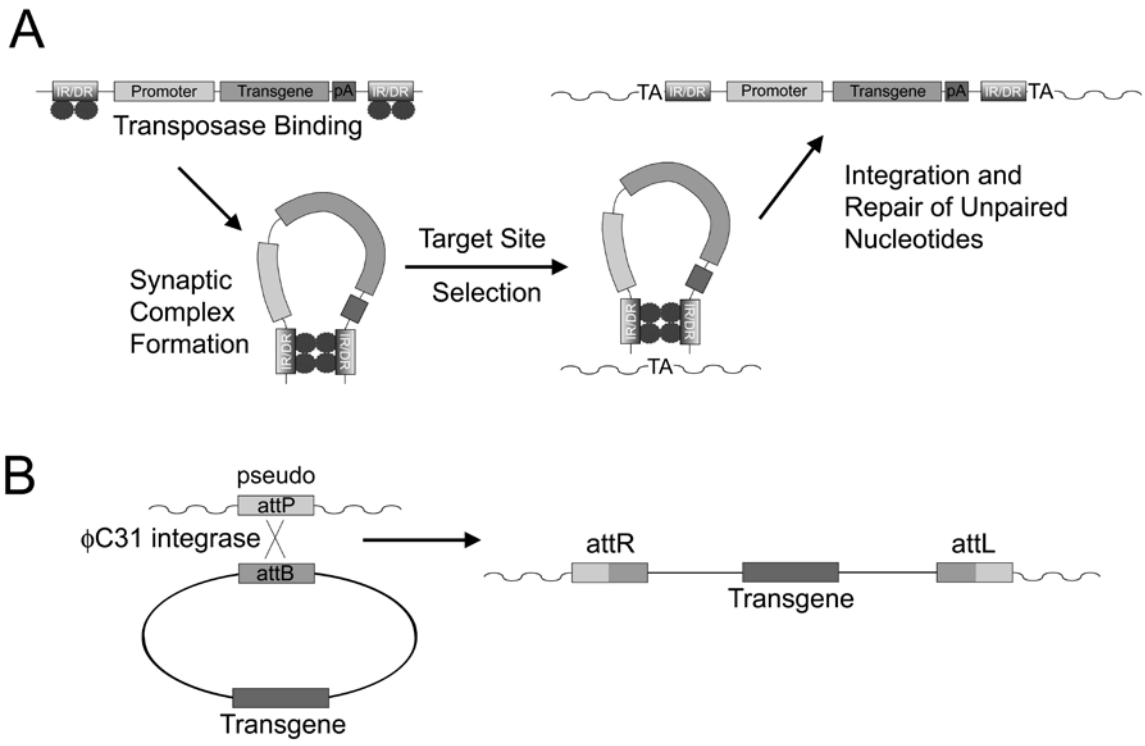

Figure 1: Comparisons of the mechanisms of integration for *Sleeping Beauty* transposon and phiC31 integrase. A. Transposition of *Sleeping Beauty* requires flanking inverted repeat/direct repeat sequences (IR/DR elements). Transposase proteins bind to the IR/DR elements and generate a synaptic complex that targets TA-dinucleotides within the genome using a cut and paste mechanism. The remaining plasmid sequence is lost during integration. B. The phiC31 integrase is a member of the serine-recombinase family of site-specific recombinases. The minimal sequences required for integrase activity are a 39 bp *attP* site and a 34 bp *attB* site [51]. The entire plasmid integrates into the genome with flanking attR and attL sites.

may lead to genomic instability. This was observed when the phiC31 integrase was intentionally expressed over time in cells in culture [55]. Providing the gene encoding the phiC31 integrase in the form of RNA may reduce these risks.

Regulatory sequences

Obtaining long-term expression without integration would be a major accomplishment for nonviral therapy. By incorporating specialized gene regulatory sequences within the plasmids to enhance expression, investigators have been able to prevent silencing and promote long-term expression without integration. By using specialized promoters [56] and incorporating other important DNA sequences such as hepatic locus control regions (LCR) [57], enhancers [58], intronic regions, untranslated regions (UTR's) [59] and polyadenylation signals, investigators can modify the levels and duration of gene expression. Utilization of these approaches for the treatment of hemophilia was first described in 2001 by Dr. Carol Miao [60]. Since that time, there has been a number of different hepatic control regions (HCR's) identified that have similar properties and can promote long-term expression. By incorporating these regions the plasmids may reside as episomes as partial hepatectomy leads to loss of expression suggesting the gene was not integrated into the genome [60]. Lastly, episomal vectors containing scaffold/matrix attachment regions (S/MARs) within the nonviral vector may facilitate long-term expression *in vivo* [61]. Proof of concept experiments using episomal vectors to treat hemophilia have been made using components of the Epstein-Barr virus [62,63], but risks of malignancy and immune rejection would prevent the use of this approach in larger animals.

Other factors that likely influence *in vivo* expression from non-integrated vectors include the CpG content of the plasmids introduced and the presence of bacterial sequences within the vector. The innate immune system can be activated by bacterial DNA and this activation is due to unmethylated CpG motifs that are recognized by the Toll-like receptor 9 on vertebrate immune cells [64]. Furthermore, CpG methylation of DNA influences transcription by altering the affinity of certain DNA-binding proteins including transcription factors and histones [65]. By generating vectors with reduced CpG content expression could be enhanced *in vivo* [66]. Furthermore, other bacterially-derived DNA sequences present within nonviral vectors alter long-term expression in vertebrates. Chen et al. has shown that making minicircles devoid of bacterial sequences can lead to enhanced and long-term expression following hydrodynamic gene delivery [67]. Recent improvements to this system make generating minicircles easier and will facilitate the analysis of other cis-acting regulatory sequences [68]. An approach to treatment of hemophilia that uses a combination of modified promoters, hepatic LCRs, S/MARs and minicircles may provide long-term expression without the concerns of insertional mutagenesis.

Localized *In vivo* tissue delivery

Localized delivery of nonviral vectors has several benefits over systemic delivery. The benefits of localized delivery may include enhanced transfection efficiency leading to a higher proportion of cells expressing the gene, reduced concern of insertional mutagenesis in off-target organs, and a reduction in the risk of generating immune responses against the introduced gene or transfected cells. For the treatment of hemophilia, two target tissues including the liver and skeletal muscle are reasonable *in vivo* targets.

Liver directed therapy is an optimal choice for gene replacement as liver sinusoidal endothelial cells and hepatocytes have the capacity to synthesize and secrete sufficient quantities of coagulation factors to affect clinical outcomes. Physical and chemical methods can be used to target the liver; however, any targeted *in vivo* method has the potential for off-target delivery. Hydrodynamic therapy is a prime example of liver-directed therapy, yet the technique still has to be perfected in larger mammals and the off-target effects have not been characterized. Ultrasound-mediated gene delivery to the liver [69] and electroporation of the liver [70] are two approaches that could be considered, yet hydrodynamic approaches are more efficacious. Chemical-mediated delivery of nucleic acids to the liver requires targeting mechanisms with many approaches involving the asialoglycoprotein receptor [15,71,72].

Skeletal muscle is an abundant target that may be used to produce and secrete functional coagulation factors and other therapeutic proteins (review [73]). Initial studies with cationic complexes revealed myotoxicity [74]; yet direct injection of naked DNA followed by electroporation was effective in treating murine models of hemophilia [75]. Further advances in the field have included chemical formulations that enhance delivery followed by electroporation [76,77], and pressure-induced transvenular extravasation [78] also known as hydrodynamic limb vein injection [79]. These improvements in skeletal muscle delivery are leading to long-term expression in muscle tissue that could translate into an attainable treatment of hemophilia in humans.

Ex vivo delivery to cells

Ex vivo gene transfer, followed by administration of the transfected cells into the recipient, is a viable approach for hemophilia gene therapy. A number of different cells types have been used to produce coagulation factors including fibroblasts, endothelial cells, hematopoietic stem cells (HSC), mesenchymal cells, and muscle cells (myoblasts). The benefits of *ex vivo* therapy include targeting a specific cell type, which essentially eliminates expression in unwanted cell types such as germline cells or antigen presenting cells (APCs). *Ex vivo* approaches are less likely to cause systemic toxicity compared to chemical-based approaches or physical methods such as hydrodynamic therapy. Lastly, *ex vivo* transfected cells may be sequestered or contained within microcapsules preventing their dissemination throughout the body and eliminating immune destruction of the producing cells [80]. This approach allows for the removal of the microcapsules if any adverse events occur. Potential drawbacks of *ex vivo* approaches include the need to individualize treatment for each patient if autologous cells are used, the labor intensive nature of the approach, insufficient protein secretion *in vivo*, and the potential for malignant expansion of the introduced cells.

Fibroblasts: Fibroblasts were the target in the first human clinical trial that used nonviral DNA as the delivery vehicle [81]. The trial involved isolation of fibroblasts from patients and stable introduction of a B-domain depleted FVIII construct using electroporation and cell selection/expansion. Twelve patients with severe hemophilia A (<1% activity) were enrolled in the trial; none with inhibitors. They received injections of expanded fibroblasts that produced FVIII into the greater or lesser omentum via a laproscopic procedure at different doses (100 to 800 million cells). Seven of nine patients who received injections into the greater omentum had either a decrease in spontaneous bleeding and/or a diminished need for factor replacement over the next several months. FVIII levels were essentially unchanged or slightly higher than baseline in all patients except one who received the highest producing fibroblast clone where levels rose by 1-4% transiently. None of the patients with injections into the lesser omentum received benefit. By one year, all patients had returned to baseline FVIII levels and their usual rate of bleeding. There were no serious adverse events or

laboratory abnormalities during the course of the trial including no evidence for immune activation or inhibitors.

Endothelial cells: Endothelial cells have an inherent capability to produce and secrete functional Factor VIII and therefore make an excellent target for gene delivery. Lin et al. reported the transfection and subsequent selection and expansion of human blood outgrowth endothelial cells (BOECs) with a FVIII expression plasmid [82]. The expanded BOECs were injected into NOD/SCID mice at various cell doses. FVIII levels correlated with injected cell dose with some animals achieving normal (100-200 ng/ml) to supratherapeutic levels of FVIII protein. Furthermore, protein levels increased overtime until the termination of the experiment at 5 months. Histological evaluation of the treated animals showed bone marrow and splenic infiltration of the BOECs. This result raises the concern that injected cells may need to be contained to prevent overgrowth or malignancy. A subsequent study used a lentiviral vector to transduce the BOECs and sequestered them within a mircrocapsule to prevent systemic dissemination [83].

HSC and Bone Marrow Stromal cells: Hematopoietic stem cells were used to produce FVIII within platelets by placing the gene under the control of megakaryocytic lineage-specific promoter. While transgenic animals and viral vectors have been used to apply this approach in murine HSC [84,85], no attempts to transfect murine HSC with nonviral approaches have been published. Similarly, bone marrow mesenchymal cells were retrovirally transduced to express FVIII and given to NOD-SCID mice resulting in phenotypic correction [86]. The ability to use nonviral approaches to stably transfect HSC and bone marrow mesenchymal cells may require further enhancements to improve efficiency, stabilize gene expression [40,87,88] and reduce the risks of transformation [89].

Myoblasts: Myoblasts have been used to produce coagulation factors and correct deficiencies in mice. The coagulation genes are typically delivered by nonviral methods followed by selection and expansion of producing clones. Initial experiments injected the transfected myoblasts back into muscles directly [90]. In an attempt to create a universal therapy for many patients, allogeneic myoblasts were used and re-implanted within mirocapsules [91]. The microcapsules prevented the destruction of the myoblasts by immune cells and prevented myoblast cell dissemination within the recipient. An extension of these studies using human derived myoblasts in mice has been conducted [92]. While this approach has potential, investigators are still optimizing the composition of the microcapsule to enhance cell viability without risking cell dissemination [80].

Concluding Remarks

Nonviral gene delivery has made significant strides over the past 10-15 years, yet challenges remain. Novel physical methods of delivery have been developed that led to significant expression within the liver. Chemical methods have evolved with newer, biodegradable and less toxic formulations. Advances have been made in enhancing gene expression over time and basic insights have been made into how expression is lost. While nonviral approaches are not being utilized in current clinical trials, they may play a role in the future. Their evolution will depend on the success of currently active AAV trials and if immune activation against AAV capsid proteins continues to be problem in human clinical trials. If viral approaches fail, attention should focus on nonviral delivery methods. Further research will be needed to identify the most promising delivery approaches in larger animals and how these can be adapted to human clinical trials. Vector design will be an important aspect of future nonviral delivery. Critical cis-acting regulatory sequences that enhance expression and promote episomal maintenance will be essential. Further improvements will include the removal of potential inhibitory sequences (bacterially-derived sequences and CpGs) and codon optimization to ensure robust expression. By optimizing these parameters, safe and effective nonviral gene therapy for hemophilia patients may be possible.

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