Non-invasive gene delivery across the blood-brain barrier: present and future perspectives

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The aging of society has arrived, and is accompanied by an increase in the absolute numbers of patients with neurological disorders, such as Alzheimer's and Parkinson's diseases (Feigin et al., 2020). Such diseases, particularly Alzheimer's disease and other forms of dementia, affect not only the patients themselves, but also the people around them, including family members and care givers. As a result, such neurological disorders are thought to carry a larger social burden compared to other diseases. The most critical point in the current situation is that there is no effective treatment despite the fact that the number of patients increase with the aging of the population. Gene therapy has great promise for the treatment of neurological disorders (Sun and Roy, 2021), but delivering therapeutic genes is a major impediment for the success of gene therapy. Nanotechnologies such as viral and non-viral vectors now permit the creation of efficient brain-targeted gene delivery systems. In 2019, the Food and Drug Administration approved Zolgensma, a gene therapy for the treatment of spinal muscular atrophy. The advent of Zolgensma confirmed that *in vivo* targeted gene therapy is a real possibility and is expected to further accelerate the development of drug delivery system technology in anticipation of gene therapy. Zolgensma involves the use of an adeno-associated virus (AAV) vector, one of the leading approaches to gene therapy, due to its high transfection efficiency; however there are issues associated with viral vectors including the production of neutralizing antibodies to the vectors and issues associated with high dose/large scale production. Regarding those points, non-viral vectors offer some distinct advantages. Non-viral delivery technologies have evolved dramatically over the past decade, especially in the use of nanoparticles in drug delivery as exemplified by lipid nanoparticles, liposomes, and micelles. The goal of this perspective is to provide a prospective look into this emerging field. To accomplish this, we mainly address three aspects of this situation: (1) brain-targeted AAV vectors; (2) non-viral delivery via non-invasive methods; (3) mechanistic studies concerning crossing the blood-brain barrier (BBB) and methodology for vector screening.

Viral vectors have been used in many clinical trials in gene therapy (Ginn et al., 2018). AAV vectors have been particularly widely used in many studies related to gene therapy, and are regarded as one of the most promising gene delivery vectors because of their positive features, such as the potential for gene transfer to non-dividing/differentiated cells, long-term expression, relatively weak immunogenicity, and, in particular, their applicability to *in vivo* gene delivery compared to other viral vectors. Additionally, AAV vectors, such as AAV9 serotypes, are heavily used to transduce therapeutic genes to the central nervous system (CNS) site via non-invasive systemic administration for the treatment of neurodegenerative disorders. Beyond AAV9, more efficient and selective brain-targeted AAV vectors are now in the progress of being developed. One example of this is AAV-PHP.eB, which was identified by a capsid selection method, Cre-recombination-based AAV targeted evolution (CREATE). The AAV-PHP.eB was reported to show at least a 40-fold greater transduction efficiency in the CNS compared to the conventional standard, AAV9. Several cell type specific/selective AAV vectors have been reported, including vectors specific for the brain microvascular endothelial cells, and astrocytes. Multiplexed-CREATE (M-CREATE) can be used to identify capsid variants that have the ability to target distinct brain cell types (Ravindra Kumar et al., 2020). It is thought that increasing cell selectivity and specificity will lead to increased therapeutic efficacy and reduced side effects. To date, multiple clinical trials involving the systemic administration of AAV vectors for the treatment of CNS diseases have been conducted. Although AAV vectors are promising and of particular interest as brain-targeted gene delivery vectors, that does not mean there are no challenges that need to be faced. There are some remaining problems in terms of the use of AAV vectors, such as problems regarding neutralizing antibodies (NAb) against AAV, biodistribution after systemic administration, and high dose/large scale production. Problems associated with the NAb against AAV and the peripheral toxicity caused by high doses are thought to be critical for the systemic administration of AAV vectors. Since AAV infections occur naturally in humans, it is possible that some patients would have pre-existing immunity. Gene expression was observed in non-human primates (NHPs) with AAV-neutralizing antibody-negative individuals, but not in positive individuals, indicating that NAb greatly affects the therapeutic effect. Considering this, the issue of whether a patient has NAb against AAV before the start of treatment usage should be demonstrated. Toxicity at high doses has been observed in several pre-clinical studies and clinical trials. The dosage for Zolgensma appears to be quite high (1.1×10^{14} vector genome (vg)/kg body weight). This dose could cause organ damage and an immune response to the viral vector because severe toxicities, such as liver damage, and the degeneration of sensory neurons, have been reported in NHPs and piglets at 2×10^{14} vg/kg body weight (Hinderer et al., 2018). Actually, toxicities have been reported for high-doses in several clinical trials such as Solid Biosciences' SGT-001 (NCT03368742) and Pfizer's PF-06939926 (NCT03362502), for the treatment of Duchenne muscular dystrophy, and even for Zolgensma. These toxicities are thought to be due to immune responses to the AAV vectors (Wilson and Flotte, 2020). Thus, avoiding harmful immune responses against AAV is essential for the development of systemic AAV vector-based treatment.

Non-viral vectors offer some advantages, including a safer and more flexible way for gene delivery, in spite of their low transfection efficiency compared to viral vectors (Ramamoorth and Narvekar, 2015). Several synthetic vectors are now available for gene delivery. There are mainly two strategies regarding gene delivery via synthetic non-viral vectors. One strategy uses therapeutic nucleic acids that are conjugated with different functional devices such as peptides, sugars, antibodies, or aptamers while another depends on encapsulating the nucleic acids in nanoparticles (NP). NP technology

has attracted considerable attention in the past decade in the field of nucleic acid delivery. It is no exaggeration to say that the era of nucleic acid nanomedicine has arrived with the approval by the Food and Drug Administration of ONPATTRO™, the first lipid nanoparticle (LNP)-based RNAi therapeutic, in 2018. In addition, LNP technology has also been applied to mRNA vaccinations for COVID-19, and the diversity of nanodesigns and the delivery of different siRNA, miRNA and DNA molecules in Phase II/III clinical trials reflects the potential of these nano-drug delivery system technologies (Herrera et al., 2018). These breakthroughs in the field of non-viral gene delivery have attracted substantial interest worldwide and clearly point to the importance of non-viral systems such as LNPs for developing more approved drugs in the future. The next step is to use these NP systems for targeting tissues other than the liver, such as the brain. Several non-viral strategies have been developed for targeting the brain using systemically injected NP systems. These include active targeting using ligand/peptide-modification, protein corona (PC), transient BBB disruption, and intranasal delivery. These strategies have been discussed in more detail in a recent review (Kimura and Harashima, 2020), so herein we focus on PC-mediated delivery. Once NPs are injected into the systemic circulation, hundreds of biomolecules bind to the surface of NPs and change the biological identity of the pristine NPs. The formation of a PC is critical for several biological events, including cellular uptake, biodistribution, clearance, immune response, and toxicity, and this process can be somewhat unpredictable at this stage. Although PC formation on a NP surface may adversely affect targeting, it is possible to control them so as to achieve more effective targeting (Chen et al., 2020). In other words, this is problematic for NPs with targeting ligands, where PC formation could mask the targeting ligands, thus leading to a reduced targeting ability and off-target effects. On the other hand, NPs that bind to factors that function as endogenous ligands can be targeted efficiently. With regard to brain targeting through low-density lipoprotein receptor (LDLR)-mediated transcytosis, it was proposed that the use of certain apolipoproteins, such as ApoE, would be useful as endogenous ligands for crossing the BBB, and for delivering a cargo to the brain using NPs with a suitable affinity for ApoE. For example, NPs coated with polysorbate-80 can bind to ApoE and enter the brain via a LDLR-mediated pathway. In another report, the liposomal surface was modified with a certain peptide, which specifically interacts with the lipid-binding domain of apolipoproteins, for manipulating the modes of apolipoprotein adsorption. When using this pathway, it should be noted that apolipoprotein-recognizing receptors are expressed, not only in the brain, but also in peripheral tissues, particularly the liver. Although no such target is known at this stage, using something like an endogenous ligand that is more brain-transferable and selective compared to apolipoproteins would be an ideal approach. It should also be noted that the quality and quantity of PC is different between static *in vitro* conditions and flow *in vivo* conditions. Thus, it would be better to directly evaluate PC *in vivo* or in an environment that mimics an *in vivo* condition, such as microfluidic channels that mimic the structure of blood vessels, in order to understand the fate of NPs.

The precise mechanism for crossing the BBB is still unknown despite the reports of several vectors, such as AAV-PHP.eB, that efficiently penetrate the BBB. Developing brain-targeted vectors based on the BBB crossing mechanism is one of the more elegant approaches. Recently, host cell factors that are involved in the gene transfer of AAV vectors have been identified by a variety of comprehensive screening and analyses. AAV-PHP.eB is the most efficient CNS-targeted gene delivery vector in rodents; however, AAV-PHP.eB cannot show CNS tropism in some mouse strains and other animal species. Using this phenomenon, a specific haplotype of the lymphocyte antigen 6 complex, locus A (Ly6a) (stem cell antigen-1 [Sca-1]) has been identified as the factor required for the AAV-PHP.eB to successfully cross the BBB (Hordeaux et al., 2019). Ly6a molecules are expressed on the surface of the BBB. The results for the transduction of AAV-PHP.eB in the absence and the presence of Ly6a showed that Ly6a facilitates binding and transduction both *in vitro* and *in vivo*. However, primates contain no direct Ly6a homolog. The question therefore arises as to how we apply this finding to gene delivery vectors for humans. It is thought that other cellular factors that share key properties with Ly6a may be prime molecular targets for gene delivery vectors in mice, NHPs, and humans. In fact, a candidate human Ly6 protein with a functional similarity to mouse Ly6a in the context of AAV BBB transcytosis has been reported (Ille et al., 2020). Differences between humans and other animal species should always be kept in mind when developing vectors, and this type of research that identifies a candidate factor for crossing the human BBB should be useful and should be accelerated. Another recent report showed that the ApoE-LDLR pathway underlies the CNS tropism of AAV-PHP.eB (Xie et al., 2021). The transduction of intravenous AAV-PHP.eB to the CNS was significantly reduced in ApoE or LDLR knockout mice compared to wild-type mice. The ApoE-LDLR pathway is generally considered to be one of the delivery routes for systemically administered non-viral NPs to the brain through the BBB, as mentioned above. It is very interesting to note that AAV vectors with a high efficiency of gene transfer to the CNS appear to essentially use the same pathway as non-viral NPs. These results are consistent with the fact that both viral and non-viral vectors largely accumulate in the liver after intravenous injection. This indicates that the therapeutic potency of both vectors can be improved by decreasing their accumulation in non-target peripheral tissues. It is expected that further molecular-level studies related to the BBB penetrating mechanism will be conducted in the future. Although the development of gene delivery vectors based on the mechanism is difficult at this stage, in fact, vectors have been developed by using various inductive screening approaches. The most advanced screening approach is considered to be the DNA barcoding approach for both viral and non-viral vectors (Adachi et al., 2014; Dahlman et al., 2017). Using this method, researchers can quantify how thousands of vectors target cells directly *in vivo* by formulating vectors that carry rationally designed DNA barcodes. To put this simply, a pool of vectors with specific DNA barcodes is administered, and the biodistribution of the vectors is quantified by deep sequencing the barcodes. This method allows hundreds of vectors to be tested at the same time using a single mouse, whereas the conventional methods require one mouse per vector. This high-throughput approach may lead to a more detailed understanding of the interaction mode between vectors and the *in vivo* environment, which would have a great impact on future research in the area of vector development. However, it should be emphasized that the key factors in this method involve ensuring that there is no interaction between vectors in the

vector pool and that the stability of the DNA barcodes does not change with the sequence.

With the rapid expansion of the concept of pharmaceutics, including regenerative medicine such as induced pluripotent stem cells and 3D printer drug discovery, gene therapy is expected to become the next innovative medicine after small molecular drugs and antibody drugs. Gene therapy is thought to be particularly promising for treating CNS disorders including Alzheimer's disease, one of the most socially burdensome diseases. A non-invasive targeted vector is attractive and would expand the range of gene therapy applications. There is no doubt that research focusing on both viral vectors and non-viral vectors for gene therapy will be further accelerated in the future (**Figure 1**). Although high-throughput screening systems such as using the DNA barcoding method are available, it will be necessary to investigate the interaction mode and response between the vectors and the body in more detail. In addition, quantitative data regarding the amount of therapeutic gene expression that are needed to cure the diseases, which is important for determining the dose needed for gene therapy and for achieving both safety and efficacy will be needed. Gene delivery research has made dramatic progress in the past decade, but there is still room for improvement, especially in delivering cargoes to the brain. Further development is still needed and desired.

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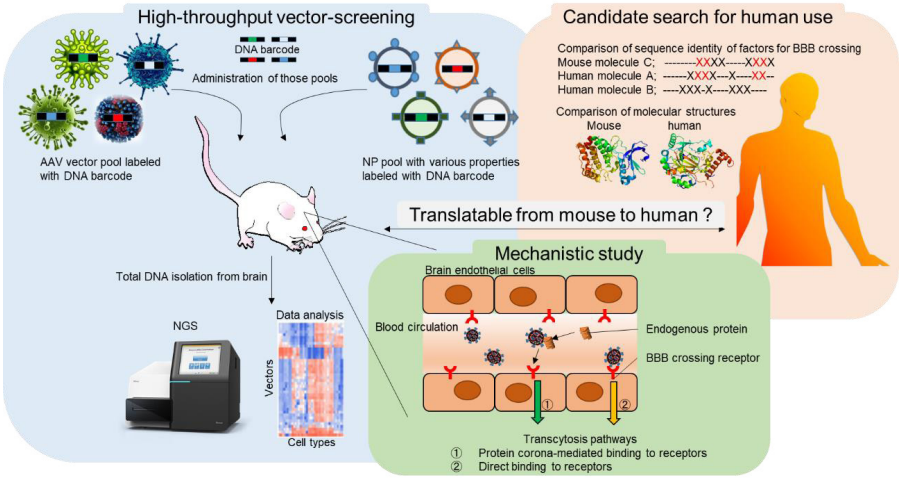

Figure 1 | **Schematic showing future prospects for the development of brain-targeted gene delivery vectors.**
Research focusing on both viral and non-viral vectors for gene therapy will be further accelerated in the future. DNA-barcoding technology can allow high-throughput vector screening. Mechanistic study on BBB permeation is thought to be important for the application from mouse to human use of the vector technologies. Differences between humans and other animal species should be kept in mind, and a candidate molecular search for human use based on the mechanistic study should be useful. AAV: Adeno-associated virus; BBB: blood-brain barrier; NGS: next generation sequencer; NP: nanoparticle.

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