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# GENE THERAPY:

## FROM SET-UP TO SCALE-UP

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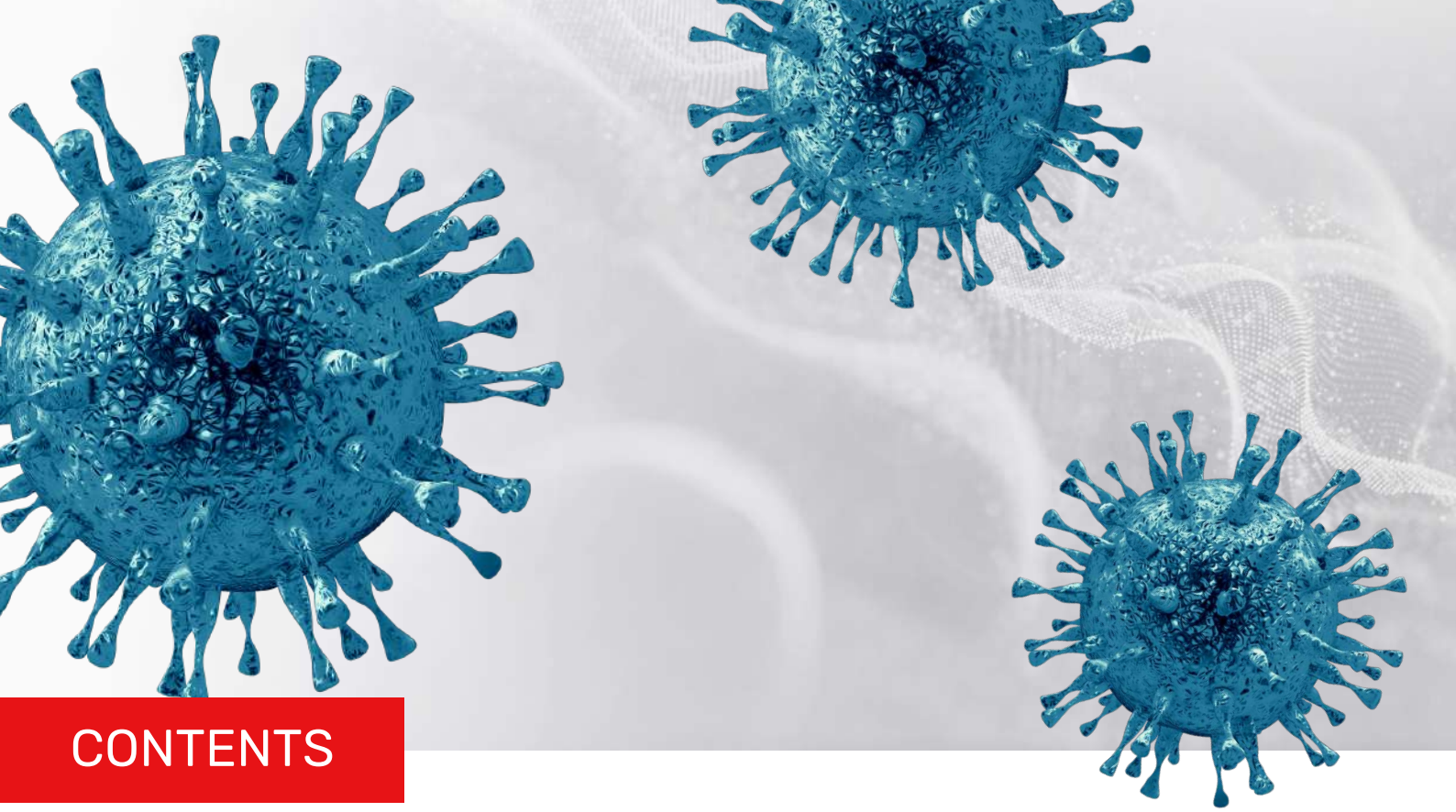

# CONTENTS



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# FOREWORD

We are pleased to present you with this eBook on the role of gene therapy: from set-up to scale-up, which has been produced by RegMedNet in association with Thermo Fisher Scientific. This eBook aims to bring you the latest developments and leading opinions from key thought leaders in the field.

Gene therapy provides a promising way to treat inherited and acquired diseases via transfer of genetic material. The advancement of viral-vector gene therapies has enabled the industry to evolve and refine manufacturing scale-up approaches to generate viral vectors for a multitude of applications and target cells. With the rapid forward movement of the field as a whole, it is critical to understand and consider variables that can affect scale up and standardization of viral vector production in the commercial setting. Different viral vectors vary in their production timelines, raw material inputs, efficiency and cost-effectiveness. Additionally, viral production platforms can be impacted by gene expression profile differences that exist in different cell lineages, and could benefit from optimization for specific cell line-dependent requirements. It is necessary to ensure that a comprehensive approach to optimizing these variables are factored in when looking at commercial scale-up and implementing viral vector production processes for therapeutic applications.

In this eBook, we delve further into accelerating gene therapy developments, as well as manufacturing solutions, the outcomes, successes, and possible pitfalls in gene therapy research.

We hope you enjoy reading about expert insights into the gene therapy workflow with us!

**Sara Mageit**
Senior Editor, RegMedNet
s.mageit@future-science-group.com

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# AAV-MAX HELPER-FREE AAV PRODUCTION SYSTEM

## STREAMLINE AAV PRODUCTION WITH A COST-EFFECTIVE, SCALABLE SYSTEM

Cost-effective, scalable adeno-associated virus (AAV) vector production is critical to meet commercial demand—and smooth scale-up to clinical production is essential. We created the Gibco™ AAV-MAX Helper-Free AAV Production System* to help reduce production costs and streamline your transition from research to clinical scale.

Make connections with an established partner.
Go to **thermofisher.com/aavmax**


* cGMP will be available with the Gibco™ Cell Therapy Systems™ (CTS™) AAV-MAX Production System.
The content provided herein may relate to products that have not been officially released and is subject to change without notice.

# Accelerating gene therapy development with complete manufacturing solutions: 60 seconds with Brandon Pence

*Brandon Pence has more than 20 years of experience in the life sciences industry leading teams in R&D, product management, marketing and business strategy.*

*Brandon began his career at Thermo Fisher Scientific in R&D and spent 15 years in a variety of technical and business management roles with increasing degrees of responsibility. Following 4 years with GE Healthcare leading their bioprocessing marketing and strategy teams at their headquarters in Sweden, Brandon returned to Thermo Fisher as the Vice President of Market Development and Strategy for the BioProduction division. In 2019, Brandon took on the role of VP and General Manager of the Purification and Pharma Analytics business and in 2021 became the VP and General Manager of the Cell Biology business.*

*Brandon is a graduate of Utah State University where he studied Cell Biology. He and his family currently reside in Utah.*

*Brandon Pence, Thermo Fisher Scientific*

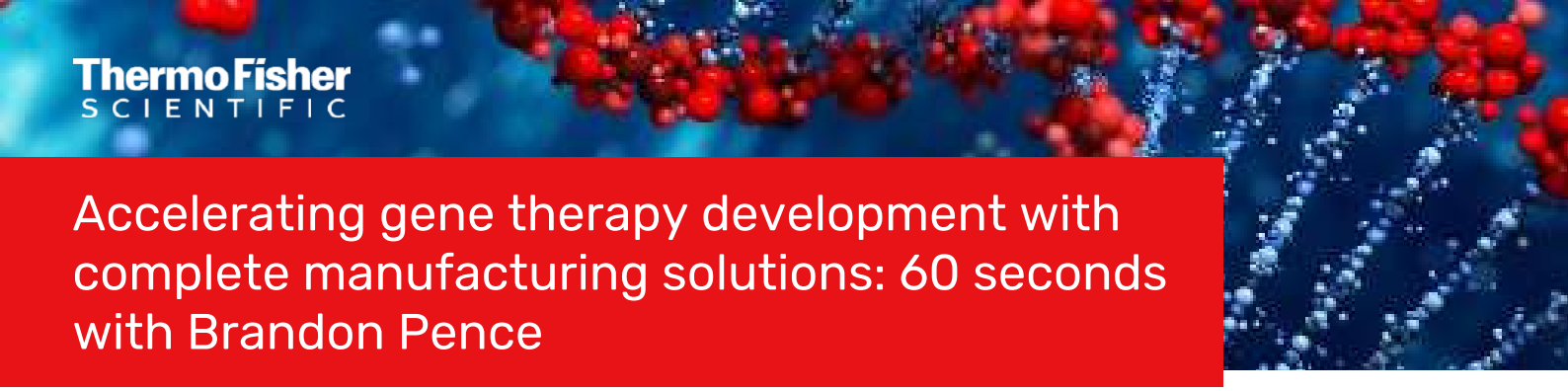
## 1 What can we do as an industry to accelerate getting gene therapies to patients?

The industry continues to focus on enabling a more seamless transition from early discovery work through process development and ultimately to commercial production. This requires us to be mindful of what the production process looks like; how can 96 well plates or other scale down systems be representative of what should happen at a larger scale? There's a lot of opportunity for improvement when looking at how we move from bench research and development to the production vessels that are ultimately supplying material destined for patients. The feedback we are getting from within the industry is that we (as suppliers) need to have the expertise to develop systems that could be the building blocks of future gene therapy manufacturing. For example, for a viral vector production platform that supports a gene therapy application, we need to consider the cell line, transfection reagents and media products all the way through the production vessel and downstream purification workflow, with the right analytics supporting each step of the process.

Every time we stop and redesign something to fit a different aspect or scale of manufacturing, we've delayed progress and possibly introduced variation in the expressed molecule. Looking back over the past 20-30 years, we need to recognize that very often what we did at the research scale didn't always translate to the production vessel, learn from it and develop better products and approaches. If we can eliminate the need for inefficient redesigns, the process of drug discovery through clinical development, clinical evaluation and then commercialization can be compressed. If we've learned anything from the past 1 or 2 years adapting to the COVID pandemic, it's that when the industry is aligned and works together, we can accelerate progress.

## 2 What lessons can be learned from the successful development and approval of Zolgensma?

One of the things that stands out to me is that it is the first approved gene therapy for younger patients – less than two years of age. Given the history of gene therapies and the fact that patient wellbeing is always at the forefront of our work, this is a major step forward in showing how safe and effective adeno-associated virus (AAV)-based therapies can be.



# Accelerating gene therapy development with complete manufacturing solutions: 60 seconds with Brandon Pence

## 3 How do "complete" manufacturing processes and technologies differ from the current standard? Why is this important for clinical manufacture of gene therapy?

A 'complete manufacturing process' includes everything from the cell line and transfection reagents used to express the vector all the way through the upstream and downstream production steps. In between cell line / vector development and the final fill / finish of the gene therapy, an enormous number of products and technologies are required to deliver something that can be utilized with a patient.

For us at Thermo Fisher, we support our customers doing this kind of work with best-in-class products and technologies, as well as through contract development and manufacturing services. For example, our acquisition of Brammer Bio a few years ago brought to us the technology and capabilities to provide viral vector process development and manufacturing services, and the ability to gain intimate knowledge of those processes so that our product teams could develop better products for that workflow. Therefore, for us, when we consider a complete manufacturing process, we are thinking about not only the right products for discrete steps, but also how a fully integrated workflow operates most efficiently.

Because of the scale and nuances inherent to viral vector manufacturing, we still have a ways to go in terms of completing and refining an integrated system. However, looking at where we are today and the investments we are making in new innovations, I'm more optimistic than ever before.

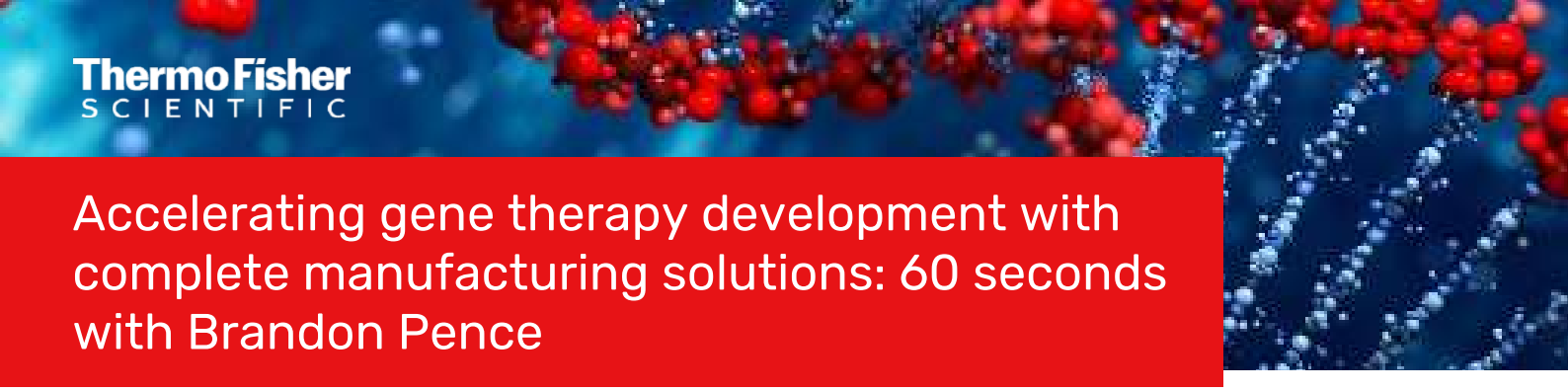
## 4 What prompted the development of the Gibco™ AAV-MAX Helper Free AAV Production System and the Gibco™ LV-MAX Lentiviral Production System? What unmet need do they address?

We are always looking for new ways to enable more successful outcomes in viral vector manufacturing processes. Our customers were consistently telling us that a lot of the products they were using, from us and from other suppliers, were not scalable or translatable into other platforms. It was clear that we were supplying customers with products that were fit for finite purposes; products which limited the customer's ability to design what worked best for them.

The idea with the AAV-MAX and LV-MAX production systems was to design optimized platforms that allow for viral vector production to move from the bench through clinical and into commercial manufacturing stages seamlessly. We optimized a system that we believe outperforms 'do-it-yourself' approaches, which we hope will drive improvements and greater consistency in their processes.

## 5 What new opportunities do the Gibco™ AAV-MAX Helper Free AAV Production System and the Gibco™ LV-MAX Lentiviral Production System offer to gene therapy manufacturers? How do these systems improve upon the current state of the art?

Both platforms have been designed to go from the bench to the production vessel; we've tried to think about what might be needed at the very smallest scale of research to the largest scale of production, and design a system that can accomplish both of those things without prohibiting optimization or customization. We wanted to avoid a system that's so rigid you can't operate outside of it, or that every time you need changes you have to go back to the



# Accelerating gene therapy development with complete manufacturing solutions: 60 seconds with Brandon Pence

drawing board. Our systems allow for optimal productivity by utilizing our established platforms, or when necessary, our technical expertise to make small tweaks that will drive optimized outcomes for our customers.

## 6 What are some common mistakes you see manufacturers making in their process development journey?

Research or drug discovery teams focus on identifying a molecule or cell line of interest so that it could be studied and analysed for clinical efficacy. This often didn't consider how that molecule or cell line might be used in a large-scale production setting if the therapy was successful, so the process development team would then design the products or process used for manufacturing. This would require looking at things like media formulations or production protocols, things that could have been included in prior research work. Then as the therapy progressed through clinical trials, they would hand the process over to the manufacturing team who would have to implement the process and determine if the supply chain and product delivery elements (e.g., format or size of raw material supply) were applicable or suitable for the production environment.

It's not necessarily a mistake, because at each stage the most important things are being focused on, but if we could enable those developers and manufacturers to be more effective and efficient in utilizing the new products and technologies now available to them, we could simplify the hand-offs and ultimately save time.

## 7 How could analytics be integrated into downstream manufacturing in the future?

This is a big component of improving or controlling process performance, especially when you think about things like empty versus full capsids in viral vector production. If we can understand what influences high quality production through analytics, that will be a significant step forward. Another important application of better analytics is safety; for example, knowing we don't have contamination from residual DNA or process impurities can enable us to move production batches to release faster and with greater confidence. Today, PCR-based assays can provide us with results in hours, not days or weeks.

A third is understanding the production system itself; using analytics to confirm a production run was successful helps us scale manufacturing or to even transfer the process to other sites. By controlling the production process and / or allowing technology transfer to occur effectively through analytics, we can take this technology worldwide in a very controlled way.

## 8 How might downstream purification evolve in the future and what part might inline analytics play?

On the purification side, Thermo Fisher is focused on ways in which we can simplify the steps between production vessel harvest to purified product, while delivering higher purity products faster. As a result of this effort, we'll continue to see improvements in the affinity capture steps of purification, including the use of novel ligands and membrane technologies. By improving our understanding of the cell culture system overall, I think we can define and design purification technologies that enable us to improve the efficiency and effectiveness of viral vector purification.

# The Viral Vector HEK Media Panel addresses HEK293 cell lineage diversity in AAV production through basal screening

## Introduction

Gene therapy has helped to address the underlying causes of previously untreatable diseases. With three approvals and more than 100 clinical trials in progress, adeno-associated virus (AAV) vectors have emerged as one of the leading gene therapy delivery vehicles [1]. The advancement of viral vector–based gene therapies has led the industry to develop and improve manufacturing scale-up to generate highly pure and potent recombinant adeno-associated virus (rAAV) vectors in HEK293 cells. However, different HEK293 progeny cell lineages vary in gene expression profiles, and adaptation can differentially impact cell metabolism, both of which may result in specific cell-dependent requirements. These differences can pose obstacles to commercial scale-up and often require considerable cell-specific optimization of media [2].

These challenges have driven the need for rAAV vector manufacturers to rapidly identify and optimize a medium specific for the different HEK293 cells and transfection processes. The Gibco™ Viral Vector HEK Media Panel, with five serum-free, chemically defined media, was developed to support rAAV vector production in HEK293 cells by helper-free triple transfection of plasmid DNA. In addition, the Viral Vector HEK Media Panel has the potential to increase viral titers independent of the manufacturing process or cell lineage. As Table 1 shows, the panel design incorporates diverse concentrations of key nutritional components to enable effective and rapid screening for improved media performance with HEK293 suspension cell lines commonly used in rAAV vector production.

Using the Viral Vector HEK Media Panel, cell growth and rAAV production were evaluated for an AAV2 serotype with two different HEK293F cell clones and an AAV8 serotype with cells adapted from the HEK293T cell lineage.

**Table 1. Heat map of Viral Vector HEK Media Panel component diversity.**

| Component | Panel medium 1 | Panel medium 2 | Panel medium 3 | Panel medium 4 | Panel medium 5 |
|---|---|---|---|---|---|
| Amino acids | | | | | |
| Vitamins | | | | | |
| Lipids | | | | | |
| Trace metals | | | | | |
| Polyamines | | | | | |

High level Low level

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## Materials and methods

### Cell culture and adaptation

HEK293F: Two internally derived suspension HEK293F clones (designated 293F1 and 293F2) were evaluated for growth and titer production. After the clones were recovered from the banked medium, both were directly adapted to each test and control medium over three passages. Test media included the five formulations from the Viral Vector HEK Media Panel, identified as panel media 1–5, and Gibco™ FreeStyle™ F17 Expression Medium (Cat. No. A1383501) was the control. Media for the 293F1 and 293F2 cells were supplemented with 8 mM and 4mM GlutaMAX Supplement (Cat. No.35050061), respectively. All of the cultures were seeded at 0.6 x $10^6$ or 0.3 x $10^6$ cells/mL every 3 or 4 days, respectively. Cells were counted using a Vi-CELL™ XR Cell Analyzer (Beckman Coulter).

HEK293T: Cells derived from adherent serum-banked HEK293T cells (ATCC, CRL-3216) were adapted to suspension in a serum-free medium. After recovery from the banked medium, the cells were sequentially adapted to the test or control medium. Test media were medium 1 and medium 5 of the Viral Vector HEK Media Panel, formulations that contain either low levels (panel 1) or high levels (panel 5) of key nutrients. FreeStyle F17 Expression Medium was the control medium. The Viral Vector HEK Media Panel and FreeStyle F17 Expression Medium were supplemented with 8 mM and 4 mM GlutaMAX Supplement, respectively. Subculturing of cells was performed twice a week with seeding at 0.35 x $10^6$ viable cells/mL for a 3-day or 4-day culture in the appropriate test medium in shake flasks. Cells were counted using a Corning™ Cell Counter.

### Transfection

HEK293F: Shake flask cultures were diluted to a density of 3 x $10^6$ cells/mL and transfected with a total of 1.5 µg/mL of plasmid DNA using PEIpro™ transfection reagent (Polyplus). The 293F1 cells were transfected at a 1:1 (w/w) DNA:PEI ratio for panel media 1, 3, 4, and 5, and a 1:2 DNA:PEI ratio for panel medium 2 and the FreeStyle F17 Expression Medium. The 293F2 cells were transfected at 1:1 for all five formulations of the Viral Vector HEK Media Panel and the FreeStyle F17 Expression Medium. The plasmid ratios (w/w) for pAAV-GFP, pRC2, and pHelper (CellBio Labs) were 1:3.03:1.44 for 293F1 cells, and 1:3:1 for 293F2 cells. Cultures were fed glucose up to a final concentration of 6 g/L at 24 hours post-transfection and harvested 72 hours post-transfection. The percent GFP-positive transfection efficiency was quantified by flow cytometry. Data reflect three experiments performed in triplicate.

HEK293T: The plasmid DNA was transfected using PEIpro transfection reagent in biological triplicate shake flasks. One day prior to transfection, cells were seeded at 1.4 x $10^6$ cells/mL. At transfection, cells were inoculated at a density of 2 x $10^6$ cells/mL and transfected with 1 µg of plasmid DNA per $10^6$ viable cells at a 1:1.5 (w/w) DNA:PEI ratio. The plasmid ratio for pHelper (Agilent), pAAV2/8, and pITR-eGFP was 1:1:1 by mass (1:1.6:1.7 molar ratio). Cells were harvested 72 hours post-transfection and percent GFP-positive transfection efficiency was quantified by flow cytometry.

### Viral genome (VG) quantitation by qPCR

HEK293F: Harvested 293F1 and 293F2 cells were lysed and diluted in Invitrogen™ DNase I Buffer. Samples were treated with Thermo Scientific™ Exonuclease I (Cat. No. EN0582) and Invitrogen™ DNase I (Cat. No. 18047019), followed by incubation with Invitrogen™ Proteinase K (Cat. No. AM2548). The AAV2 VG titer was quantified by qPCR using an Applied Biosystems™ TaqMan® Assay (Cat. No. 4332079) targeting GFP. Linearized pAAV-GFP was used to generate the standard curve.

HEK293T: AAV8 was harvested after cell recovery and lysis. Prior to DNase treatment, the lysate was treated with Benzonase™ Endonuclease (MilliporeSigma). The extracts were then treated with DNase, and viral DNA was purified using the High Pure Viral Nucleic Acid Kit (Roche Diagnostics GmbH). The VGs were quantified by qPCR using a primer–probe set specific for the *egfp* gene. The pITR-eGFP plasmid was linearized and used to generate the standard curve for quantification.

### Total particles (TP) quantitation by ELISA

HEK293T: The AAV8 fraction particles were quantified using the AAV8 Xpress ELISA assay (PROGEN Biotechnik GmbH). This sandwich ELISA recognizes a specific surface epitope on the assembled capsid via a conformational change that is not present on unassembled capsid proteins. The TP was calculated using a 4-parameter logistic (4PL) regression. The percentage of full capsids (vs. empty capsids) was calculated by dividing the calculated TP by the VG titer.

Note: HEK293T growth and AAV8 productivity evaluations of the Viral Vector HEK Media Panel were conducted by the Institute of Experimental Biology and Technology (iBET, Portugal).

## Results

### HEK293F cell growth and AAV2 productivity

Prior to transfection, the population doubling times for the 293F1 and 293F2 cells were determined to evaluate and compare growth obtained using the Viral Vector HEK Media Panel and the control, FreeStyle F17 Expression Medium. The 293F1 and 293F2 clones demonstrated comparable average doubling times with all five media formulations, relative to FreeStyle F17 Expression Medium (Figure 1). These results indicated the Viral Vector HEK Media Panel formulations did not significantly alter cell growth and would support sufficient growth for productive AAV2 transfection.

The HEK293F VG results revealed that the 293F2 clone produced higher overall average titers than did the 293F1 clone, with all media, as shown in Figure 2. However, both clones produced the highest average VG titers with panel media 4 and 5, compared to FreeStyle F17 Expression Medium, with the 293F1 cells demonstrating 10-fold higher titers and 293F2 cells demonstrating 2-fold higher titers (Figure 2A and 2B).

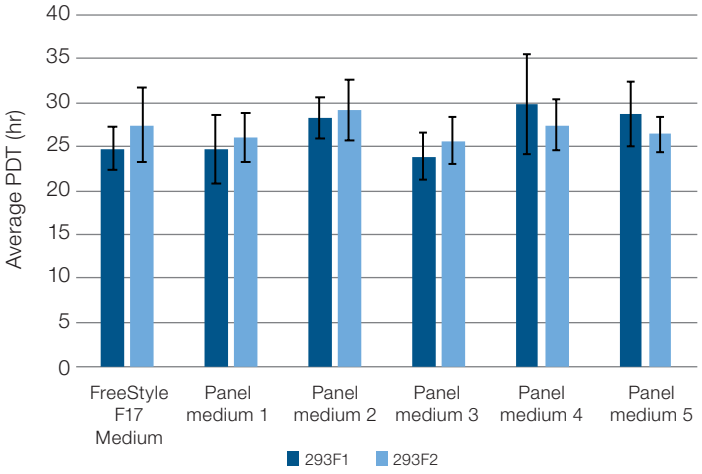


**Figure 1. HEK293F population doubling time.** The 293F1 and 293F2 cell clones had comparable average population doubling times (PDT) in the five Viral Vector HEK Media Panel formulations and FreeStyle F17 Expression Medium.

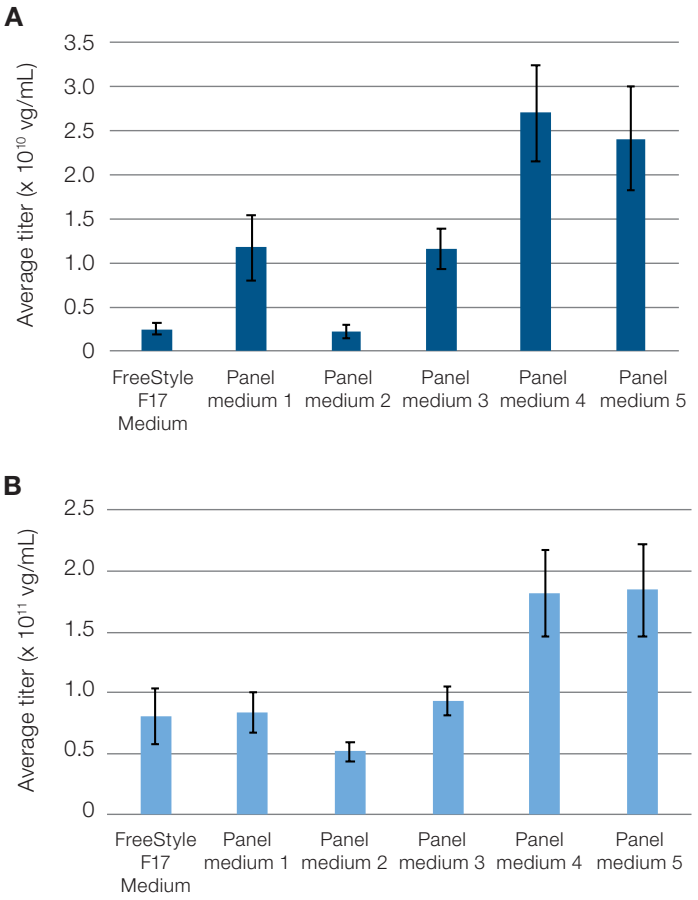


**Figure 2. HEK293F AAV2 viral genome titers. (A)** The 293F1 cells produced 10-fold higher average AAV2 titers with panel media 4 and 5, compared to with FreeStyle F17 Expression Medium. **(B)** The 293F2 cells demonstrated 2-fold higher average titers with panel media 4 and 5, compared to with FreeStyle F17 Expression Medium. (Data reflect three experiments performed in triplicate.)

### HEK293T cell growth and AAV8 productivity

The HEK293T population doubling time in panel media 1 and 5 was comparable to that in the control medium, FreeStyle F17 Expression Medium (23 ± 1 hour) (data not shown), again suggesting that the panel media formulations did not negatively impact cell growth and would support sufficient growth for productive transfection.

The HEK293T AAV8 viral genome titer results were comparable with panel media 1 and 5, compared to FreeStyle F17 Expression Medium (Figure 3A). Determination of full capsids revealed that Viral Vector HEK Media Panel 1 produced an average of 76% full capsids, compared to 51% and 40% with the control medium and panel medium 5, respectively (Figure 3B).

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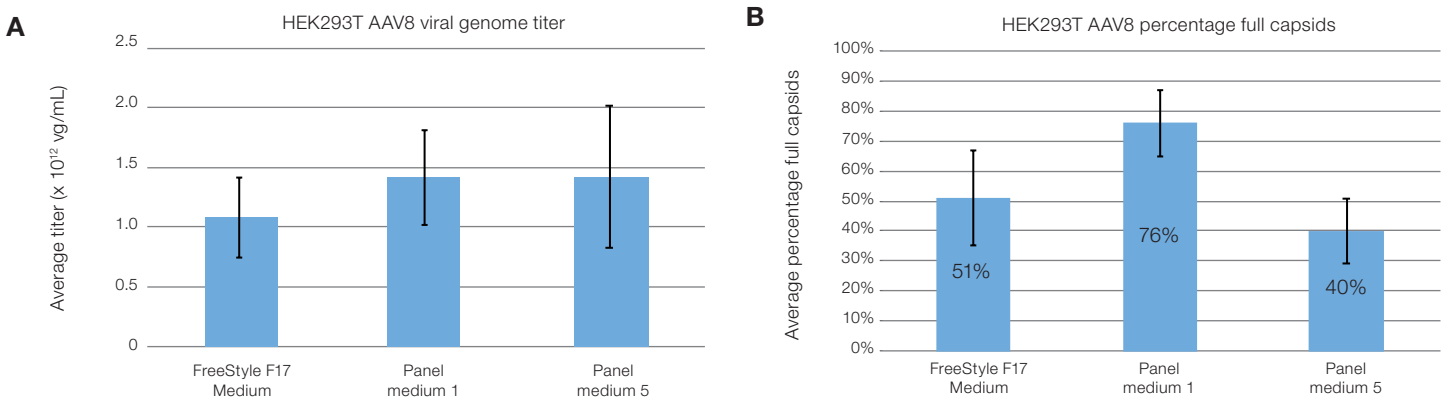


**Figure 3. HEK293T AAV8 viral genome titer and percentage full capsids. (A)** Panel media 1 and 5 yielded comparable average titers when evaluated with FreeStyle F17 Expression Medium. **(B)** Analysis of the average percentage of full capsids showed that panel medium 1 produced 76% full capsids compared to 51% with FreeStyle F17 Expression Medium and 40% with panel medium 5. (Testing was conducted in biological triplicate.)

## Conclusions

AAV manufacturers need to rapidly identify candidate media formulations that can be adapted to their HEK293 cell lines, various AAV serotypes, and transfection processes. Regardless of the HEK293 lineage or clones tested, the Viral Vector HEK Media Panel formulations did not significantly alter cell growth compared to the control, FreeStyle F17 Expression Medium.

Evaluations with the 293F1 and 293F2 clones showed the media panel yielding differential clone-dependent titer responses, respectively demonstrating 10-fold and 2-fold titer increases for panel media 4 and 5 compared to the control, FreeStyle F17 Expression Medium. These findings suggest the potential for further evaluation and optimization of panel media 4 and 5 for the HEK293F cells.

Results with the HEK293T cells demonstrated AAV titer production with panel 1 and 5 was comparable to FreeStyle F17 Expression Medium. In addition to titer production, the percentage of full capsids was evaluated to assess the quality of the AAV product. The evaluation of full versus empty capsids is often considered because empty capsids are a manufacturing impurity that can affect the efficacy and safety of the AAV vector products [3]. The results of this testing revealed an average of 76% full capsids with panel medium 1, compared to 51% and 40% with FreeStyle F17 Expression Medium and panel medium 5, respectively. These results suggest the potential for further analysis and optimization of panel medium 1 for the HEK293T cells.

The Viral Vector HEK Media Panel has demonstrated the potential to address production challenges by enabling rapid screening of media candidates that support increased titers and higher-quality AAV production with diverse HEK293 cell lines. With the rapid changes in AAV manufacturing platforms, the media panel is also poised to enable further process development through formulation optimization.

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# Gene Therapy Roadmap

Your Gene Therapy Roadmap from production to commercialisation.

End-to-end gene therapy solutions to support your viral vector workflow.

Advancement in gene therapies offer great promise to treat life-threatening diseases. With Thermo Fisher Scientific, you have the support you need to push your therapeutic development strategies a step further. We offer a suite of scalable and robust solutions for all stages of the gene therapy workflow, from development through to commercialisation, to help you accelerate your development and meet regulatory standards with confidence.

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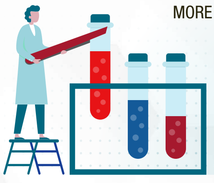

## 6 ANALYTICAL TESTING

Detection of impurities and contaminants is critical in gene therapy production. Doing so mitigates risk and prevents extensive production impacts. We provide rapid analytical assays with total workflow solutions to align with regulatory requirements, from evaluation through to validation and approval.

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Connect with us to bring your gene therapy to commercialisation with confidence

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RESOURCES

# Optimizing AAV Manufacture

**With the potential to cure genetic disorders rather than alleviate symptoms, gene therapies look set to revolutionize the field of medicine. Adeno-associated viruses have emerged as the vector of choice for delivering therapeutic genes to target cells, but manufacturing processes need to be improved and optimized to unlock their full potential.**

*By Orjana Terova and Zoltan Gulyas*

Over the last decade, significant progress has been made in our ability to deliver therapeutic genes to target cells. Medicines that are able to replace faulty and missing genes are genuinely life-changing.

Despite the relative immaturity of the field, two gene therapies have already been approved by the FDA (Luxturna® and Zolgensma®), and the pipeline looks strong; the FDA expects that approvals for cell and gene therapy products will rise to 10–20 per year by 2025. Right now, gene therapies are targeting orphan diseases, especially in children, but they have the potential to treat central nervous system related disorders, such as Alzheimer's, Parkinson's or Huntington's disease.

Expectations are high, but there are many challenges on the road ahead; as gene therapies are looking to treat larger patient populations, there is a concomitant need to increase the manufacturing scale, and improve productivity and process control.

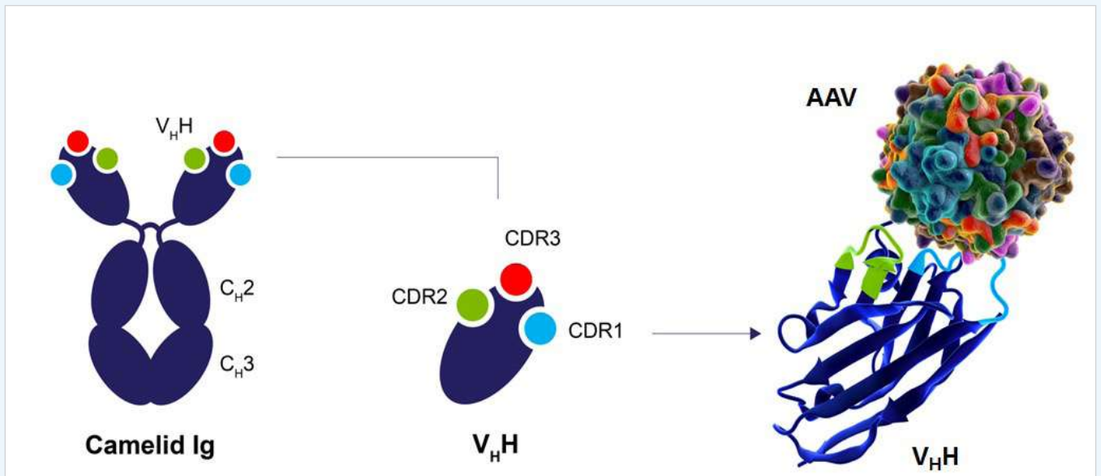
*The vector of choice*

Selected viruses have been successfully engineered into smart vehicles to deliver DNA to target patient cells. These viral vectors lack any viral genes but contain DNA sequences of interest for various therapeutic applications. In particular, recombinant adeno associated viruses (AAVs) have emerged as the vector of choice for many therapies for several reasons.

First and foremost, AAVs are generally considered safe, as they are non-pathogenic and non-toxic, and have inherently low immunogenicity, when compared with other viruses. Scientists have identified 13 naturally occurring serotypes so far, and each of them has different tropism (i.e., ability to target specific cell types), which enables selective transduction of specific tissues and organs. Companies are actively developing novel, engineered capsids to further improve tropism, and thereby increase the potency.

From a more practical standpoint, AAVs are relatively simple to manufacture, and these vectors have no lipid envelope as found on retroviruses and lentiviruses, so they are more stable, and able to withstand the typical process conditions used for protein purification such as low pH and high salt.

The potential of AAVs to treat wider patient populations and target more common disorders is somewhat limited due to the manufacturing and scale-up related challenges. The majority of gene therapies in late clinical phases are the result of first-generation processes that started as research projects in academia or hospitals years ago. In these settings, vector production is often performed by the classical tools and methods such as adherent cell cultures on plates or cell factories, sonication for cell disruption and ultracentrifugation for purification. Most of these techniques are either not scalable or can only be scaled out.




CaptureSelect technology is based on the single N-terminal domain of Camelid IgG, the $V_HH$ fragment. Camelid-derived immunoglobulins are naturally devoid of light chains. The small size of the $V_HH$ fragments allows for binding to epitopes of the target molecule which are difficult to access by larger immunoglobulins. Overall, the $V_HH$ fragments offer high specificity, affinity and stability.

## Realities in the Field

Our dedicated team of field applications specialists are more than happy to answer questions and help solve problems. Here are some advices and points to consider regarding affinity capture of AAVs.

1. Process steps between harvest and capture chromatography are often neglected or not properly optimized, but the feed-stream quality can have a profound impact on purity, yield and process performance. Removal of all insoluble components by depth and membrane filters is important to avoid backpressure issues and column clogging. We also recommend soluble impurity reduction by various techniques (such as endonuclease treatment, flocculation, tangential flow filtration, and/or various chemistries on solid support) as much as possible prior to affinity capture.
2. Low product concentration in the load can cause earlier break-through and thereby resin capacity loss. Feed-streams can be concentrated by TFF, which also provides impurity clearance and reduces the loading time.
3. If capsids are present in the flow-through, increasing the residence time may be able to mitigate this.
4. Root causes for low recovery of the capture step could be caused by under-loading the column due to insufficient product quantities (loading around 1E12 vg/mL resin or below, which is 2-3 logs lower than the AAVX capacity), lack of elution efficiency, and/or overestimation of load concentration. Column volume reduction, eluting in upflow and optimizing the elution (evaluating different buffers, pH and additives) can mitigate the product loss.
5. Insufficient eluate purity can be resolved by incorporating and optimizing intermediate washes between load and elution.
6. If the affinity resin is meant to be reused, cleaning optimization should be performed to avoid carry-over issues. We recommend an acidic strip followed by cleaning with a chaotropic agent, such as guanidine hydrochloride. Concentrations and contact times are process dependent, but upflow direction is always recommended. Please note that our AAV affinity resins are compatible with up to 25mM NaOH only.

*Essential evolution*

Today's gene therapy developers are using scalable techniques from the beginning of development, and recognize the need to not only improve productivity, but also process robustness and reproducibility. At the same time, regulatory agencies are expecting increasingly established product control and product characterization.

The monoclonal antibody (mAb) field was in a similar position not so long ago. It, too, had to evolve and mature, and can serve as a "role model" to the gene therapy field. Today, suspension cultures are used with high cell density to achieve high titers, and purification is performed by multiple chromatography steps including the highly selective affinity capture and the orthogonal polishing steps. All tools and methods are GMP-compliant, and the product is extensively characterized to ensure its safety and efficacy.

Broadly speaking, the gene therapy field needs to follow a similar path, which requires significant investment of time and resources, while meeting speed-to-market needs. Though the path is similar, we must recognize that we cannot simply "copy and paste" solutions from the biopharma industry as these were designed with a different mind-set for different molecules. When it comes to optimizing AAV processes, there is much work to do.

In terms of upstream processing, gene therapy developers are still seeking reproducibility and looking to push productivity orders of magnitude higher than the current standards. Improved packaging mechanisms are also needed to boost the percentage of full capsids (those that contain genetic material)

## CaptureSelect and POROS Up Close

CaptureSelect technology is based on a strong foundation of over 15 years of experience in developing affinity ligands and producing resins for GMP manufacturing. The platform uses the variable domain of the heavy-chain-only camelid antibodies called $V_HH$ – a single domain with a size of 15 kDa that provides full functionality in antigen specific recognition and high affinity binding. Their compact structure and the lack of light chains also results in increased stability, which allows them to withstand a wide variety of process conditions when applied as affinity ligands.

For large target molecules such as AAV, the CaptureSelect ligands are immobilized on the POROS backbone, which is a rigid, polystyrene-divinylbenzene based solid support with large pore structure to ensure high binding capacity and a more efficient purification process. The large pore structure of the POROS resins results in reduced mass transfer resistance and as linear velocity increases, capacity and resolution decline very little. This leads to improved process productivity.

## Innovation for All

In Thermo Fisher Scientific's BioProduction Division, all business units are devoted to bring solutions to the gene therapy field. The cell culture team is focused on the development of suspension cell lines, media and additives to ensure high productivity upstream. The single-use team develops bioreactors for suspension culture (both in batch and continuous mode), and highly-customized single-use bags that are gamma irradiated for immediate use in clean rooms for closed processing. The purification team is focused on highly specific affinity resins to establish platform capture for AAVs. We also offer ion exchange resins to enable full capsid enrichment and additional impurity clearance. Finally, the pharma analytics team have developed highly sensitive assays for process-related impurity and advantageous agent detection. We have just launched a residual DNA detection kit for HEK-293 cells, and an Sf9 specific kit is in the works. Lastly, our dedicated viral vector services team has extensive expertise in clinical and commercial manufacturing of AAV, to progress programs from early to late phase development and commercialization.

versus empty capsids (which have no therapeutic value).

Turning our attention downstream, developers need scalable purification methods with high selectivity towards the molecule of interest, and high recovery to make sure not to lose the produced material.

Surrounding these elements is the need for accurate and reliable analytics that enable DoE-based process development, product characterization and quality control.

Recognizing the need for progress across the board, the Thermo Fisher Scientific bioproduction team is active in all these areas (see sidebar: "Innovation for All").

*AAV Affinity Chromatography – the game changer*

Focusing on purification, many companies have moved away from ultracentrifugation over the past few years and established multiple chromatography steps including ion-exchangers and hydrophobic interaction resins to achieve the required purity. In addition to the lengthy processing time and raw material cost, however, such multi-step processes generate cumulative yield losses. Moreover, process development lead times increase, hindering speed-to-market.

Affinity chromatography can overcome most of these challenges, as it can selectively capture the product of interest from crude material, providing high purity and yield in a single step, and robust methodology with less need for process optimization. This highly specific separation delivers significant improvements to downstream processing by reducing the number of purification steps and maximizing productivity.

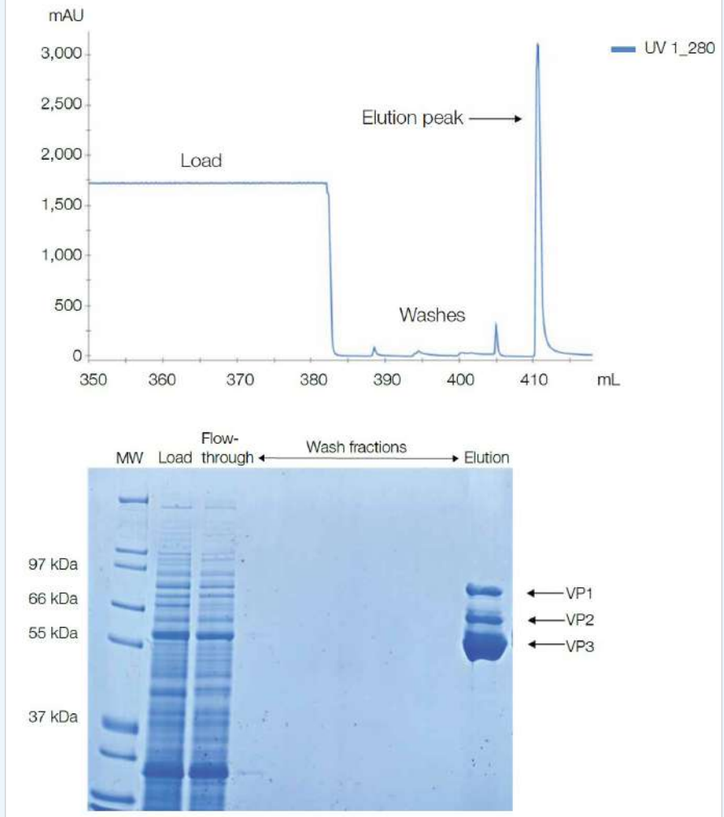
Affinity chromatography is already a key element of the purification platform for monoclonal antibodies (consider Protein A), and specifically from Thermo Fisher Scientific's standpoint, our CaptureSelect™ team has been developing affinity solutions for over 15 years, enabling a similar paradigm shift in the purification of antibody-derivatives, recombinant proteins and now viral vectors. Due to the larger size of viral vectors, the affinity ligands are immobilized on Thermo Scientific™ POROS™ base beads, which are extremely suited for the purification of larger molecules (see box: CaptureSelect and POROS Up Close).

We currently offer three POROS CaptureSelect AAV affinity resins – AAV8, AAV9 and AAVX. As their name suggest the POROS CaptureSelect AAV8 and AAV9 resins were developed for the indicated serotypes, while the POROS CaptureSelect AAVX resin works for all naturally occurring serotypes as well as engineered capsids. This allows our customers to use it as a platform capture step in all their AAV projects (similarly to Protein A for mAbs). Based on the feedback we received since it launched, the AAVX resin is largely fulfilling the industry's expectations, enabling high purity in a single step, offering process consistency from lab to production scale. Lastly, the performance of POROS CaptureSelect AAV affinity resins is maintained even at high flow rates, thereby enabling increased productivity and process flexibility.

*Team players*

Even when using affinity chromatography, users still need to perform process optimization to ensure high purity and recovery. This work is more crucial in the gene therapy processes, where the current product and process understanding is limited, and the "plug and play" approach often leads to lackluster process performance. The importance of optimization goes beyond the affinity capture step (see sidebar: "Realities in the Field").

In this rapidly evolving and challenging field – and with such high expectations – teamwork is more important than ever. Upstream, downstream and analytical experts need to be in constant communication, and combine efforts to move the needle. This is why our team of field application specialists is keen to engage and collaborate with gene therapy developers on technical matters – to discuss recommended conditions, troubleshoot problems, and brainstorm on challenges. We are eager to learn, and as our knowledge base grows, we become better equipped to provide more efficient support and develop next-generation solutions that can help companies overcome the productivity and scalability challenges.




Chromatogram showing elution peak of rAAV6 purified on POROS CaptureSelect AAVX affinity resin (top). Fractions from AAV6 purification run on a Coomassie stained gel. The capsid proteins VP1, VP2, and VP3 are indicated (bottom).

At Thermo Fisher Scientific, we are committed to provide the tools and services needed to manufacture AAV drug products, smoothing the path to commercialization and helping to bring life-changing gene therapies to the clinic faster.

*Orjana Terova is Senior Product Manager, Purification, and Zoltan Gulyas is Senior Field Applications Specialist, Purification, both at Thermo Fisher Scientific.*

Review

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Regenerative Medicine

# The role of gene therapy as a valuable treatment modality for multiple spinal pathologies

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The world of biomedical research has led to several breakthroughs in the treatment of various spinal pathologies. As we investigate chronic pathologies of the spine, we start to unravel the underlying molecular mechanisms through a careful analysis of mutated genetic sequences. Investigations have led to gene therapy being explored for its potential as a treatment modality. Despite only about 2% of current gene therapy trials being centered for spinal pathologies, spinal diseases are valuable targets in gene therapy administration. Through a comprehensive literature review, our objective is to discuss the molecular mechanisms behind gene therapy for spinal pathologies, the genetic targets, along with the outcomes, success, and possible pitfalls in gene therapy research and administration. The emerging development of robotic technologies and intelligent carriers are recognized as a promising innovative technique for increasing the efficiency of gene therapy and potentially resolving spinal pathologies.

First draft submitted: 29 September 2020; Accepted for publication: 23 February 2021; Published online: 12 March 2021

**Keywords:** cell therapy • gene therapy • growth factors • regeneration • repair

With the discovery of transduction in bacteria, scientists in the early 20th century hypothesized of how the property of genetic transfer could be applied to human genetic diseases [1]. Being that clinical diseases are related to genetic disruptions, this idea was promising. Decades later, scientist have discovered how to transfer genetic material into patients' genomes via vectors such as viruses, nucleic acids and genetically engineered microorganisms. These profound discoveries have led to the emergence of gene therapy, a technique that modifies disease-causing or malfunctioning genetic material and replacing it with functional genetic material that corrects or replaces the mutated/disease-causing genetic sequence. Gene therapy provides a way to treat inherited and acquired diseases via transfer of gene material and its associated regulatory elements (plasmid) into patients. These techniques pioneered a host of clinical trials ranging from protein deficiency disorders, monogenetic deficiencies, to cancers [2]. The varying success of these trials is a result of the vector used, nontargeted systemic effects of the disease, and modes of administration. Although the use of gene therapy has increased significantly within the last decade, only 2% of gene therapy is centered on spine disorders [1]. However, with the ongoing success of gene therapy in other fields and the promise that gene therapy can replace invasive procedures, we can expect a blossoming of its usage in spinal treatments.

By definition, spinal disorders are conditions that compromise the functional and structural integrity of the spinal cord. The majority of spinal disorders are ideal for gene therapy because they are localized and monogenetic. Spinal disorders remain a formidable problem that can result from acute traumatic injuries or chronic degenerative process. In this review, we will focus on current applications of gene therapies on various spinal disorders such as disc degeneration disease, spinal cord injury (SCI), tumors and scoliosis. The neuromuscular disorder, spinal muscular atrophy (SMA), will also be discussed for its current application and its potential gene targets. In addition,

Future Medicine

we will analyze the genetic components to errors in embryological development while further addressing the future applications and expectations surrounding gene therapy in spinal care.

## Gene delivery

The development of gene delivery vectors began in the 1990s with the use of replication defective adeno-associated virus (AAV) [2]. These viruses were the first molecular tool that enabled efficient and low toxic transfer of genes into human somatic cells. They are engineered from nonpathogenic and nonenveloped parvovirus that are predominantly nonintegrating. Its success was marked by improvements in patients with SMA, congenital blindness and hemoglobinopathies [2]. However, their low DNA carrying capacity (~5 kb) and lack of specificity and integration led to the use of lentiviral vectors. These are retroviruses that integrate into coding regions of DNA, improve the gene transfer into nondividing cells, and increase the carrying of larger and more complex gene cassettes. However, viral vectors are still limited to gene modification as they are restricted to only mediate gene addition capabilities.

Nonviral vectors are simpler systems that avoid the insertional mutagenesis and immunogenicity of transduced cells seen in viral vectors. Liposomes, naked DNA, oligonucleotides and transposons are some of these nonviral vectors [3]. In addition to inserting genes such as viral vectors, nonviral vectors can edit the genome. Genome editing is a more precise way of repairing disease-causing genes than the conventional gene therapy approach of gene addition. The genome editing technologies are based on engineered or bacterial nucleases that can insert, delete and alter gene sequences in a site-specific manner [2]. Examples of these genome editing techniques include zinc finger nucleases, transcription activator-like effector nucleases and clustered regularly interspaced short palindromic repeats Cas9 nucleases [4]. These site-specific integration systems require biomaterials such as lipids, polymers and peptides to pack nucleic acids into nanoparticle or hybrid systems for delivery. With a large scale of production and low host immunogenicity the biocompatibility, safety and success of nonviral vectors is far beyond viral vectors [5]. However, low levels of expression and transfection of genes are still barriers in these vectors. Recent advances in vector technology will in time address these issues.

In terms of the delivery mechanism, gene delivery remains a challenge in the field as it is highly dependent on the vector, the desired location of treatment and minimization of side effects. There is a diversity of gene delivery methods that include injection, oral, intranasal, pulmonary, dermal, ocular, vaginal, rectal and optic forms. To improve the bioavailability, new physical or electrically driven techniques such as electroporation and sonication are employed. Regardless, needle delivery is still very common in spinal disorders due the anatomical factors involved. Needle delivery includes intravenous, intramuscular, intrathecal and intraneural injection [6]. The preferred route is decided based upon factors that include the barriers (blood–brain and blood–nerve barriers) the vectors must cross to reach the target tissue and the level of expression that would improve pathophysiology [6].

## Spinal applications of gene therapy

### Spinal cord injury

SCI can be a devastating and life-altering injury depending on the severity and deficits encountered [7]. It is widely established now that injury occurs not only in the primary mechanism (such as direct impact), but also from subsequent secondary insults. Treatments have been aimed at stabilization and halting progression of injury, as well as mitigating the secondary effects such as increasing perfusion, ensuring adequate nutrition/caloric intake, etc. Through increased research in the molecular basis of SCIs, gene therapies are being explored as a treatment model for this pathology.

Several studies have revealed mechanisms where alterations in molecular activity could improve SCI injuries. In a study by Franz *et al.*, neurotrophic factor delivery to the epicenter of SCI demonstrated response with enhanced neuronal survival and axon growth [8]. NT-3 delivery was found to promote axonal regeneration, indicating its significance. (Tables 1 & 2) [8]. In addition, *BDNF* gene delivery to injured spinal cord can act over extended distances to amend neuronal degeneration as was shown in rodents and nonhuman primates (Tables 1 & 2) [8]. In another study, Hu *et al.* targeted *PAX2* using miRNA to evaluate motor deficit following SCI [9]. *PAX2* is found broadly expressed in the intermediate region of the spinal cord and is involved in the development of interneurons, neurotransmitter transmission, axon morphology and dendritic arborization control (Table 1) [9]. As such, it was hypothesized that miR-362-3p may serve as new therapeutic route in treating neuropathic pain following SCI through *PAX2*, which was verified as target gene through luciferase assay [9]. In rat models, they found that overexpression of miR-362-3p improved function, decreased neuronal apoptosis and neural inflammation in

**Table 1. Brief overview of the molecular targets, delivery methods and vectors or techniques used to administer gene therapy among common spinal pathologies.**

| Indication | Molecular targets | Delivery method | Delivery vector/technique | Ref. |
|---|---|---|---|---|
| SCI | NT-3 | - Intraspinal injection; spinal cord epicenter of SCI<br>- Muscle injection | - Lentivirus<br>- AAV | |
| | BDNF | Intraspinal injection; spinal cord epicenter of SCI | Lentivirus | |
| | PAX2 | iv. | miRNA administration | |
| | CNTF | - Cortical injection<br>- Intraspinal injection | - AAV | |
| | GDNF | - Intraspinal injection; spinal cord epicenter of SCI | -AAV | |
| Spinal cord tumors (16) | BRAF<br><br>pHSV-TK | - *In vitro* transfection<br>- Intraspinal injection; tumor lesion<br>- *In vitro* injection into rats | - *Salmonella typhimurium* and hyaluronan administration.<br><br>- Nonviral gene carrier PgP | [10] |
| DDD (4) | mTORC1/RAPTOR/RICTOR | *In vitro* human disc nucleus pulposus cell | Nonviral RNA interference (siRNA) | [11] |
| | TNF-α, IL-1β/TNFR1/IL-1R1 | *In vitro* human disc nucleus pulposus cell | Lentiviral CRISPR/Cas9 | |
| Scoliosis | SLC39A8 (37)<br>*PPP2R3B (36)*<br><br>*FBN1* | - *In vitro* injection into zebrafish<br><br>- *In vitro* injection into *Drosophila* | - CRISPR/Cas9<br><br>- RNAi | [12,13] |
| SMA | - Alternative splicing of functional SMN2 pre-mRNA;<br>- Nusinersen®†<br>- Risdiplam®<br>- Branaplam® | - Intrathecal injection (nusinersen)<br>- Oral administration (risdiplam and branaplam) | ASO (nusinersen) | |
| | Functional SMN1 replacement; Zolgensma®† | iv. injection | - Onasemnogene abeparvovec<br>- (previously called AVXS-101 and scAAV9.CB.SMN) | |
| Spina bifida | BDNF<br>Combined BMSC and CRMP4 siRNA | - Intra-amniotic injection<br>- Intraspinal injection | AAV | |

†US FDA approved.
AAV: Adeno-associated virus; ASO: Antisense oligonucleotide; BMSC: Bone marrow-derived mesenchymal stem cell; CRISPR: Clustered regularly interspaced short palindromic repeat; DDD: Degenerative disc disease; IL-1R1: IL-1 receptor 1; iv.: Intravenous; PgP: Poly (lactide-co-glycolide)-graft-polyethylenimine; pHSV-TK: Plasmid-encoding herpes simplex virus-thymidine kinase; ScAAV9: Nonreplicating AAV capsid; SCI: Spinal cord injury; SMA: Spinal muscular atrophy; SMN: Survival motor neuron; TNFR1: TNF receptor 1.

**Table 2. The major findings of studies that use gene delivery methods to improve spinal cord injury.**

| Study (year) | Major finding | Ref. |
|---|---|---|
| Franz *et al.* (2012) | - NT-3 delivery promoted neuronal survival and axon growth in SCI models<br>- BDNF gene delivery also showed ability to amend neural degradation | [8] |
| Hu *et al.* (2019) | Overexpression of miR-362-3p showed improved function, and decreased neuronal apoptosis in SCI rat models | [9] |
| Hodgetts *et al.* (2018) | CNTF delivery via AAV promoted axonal survival | [14] |
| Mukhamedshina & Shaymardanova (2016) | UCB-MCs and direct GDNF delivery resulted in improved motor function in rats with SCI | [10] |

AAV: Adeno-associated virus; SCI: Spinal cord injury; UCB-MC: Umbilical cord blood cell-mediated.

those with SCI [9]. Furthermore, the miRNA alleviated neuralgia and reduced activation of ERK and p38 through inhibition of *PAX2* [9].

As previously mentioned, one of the well-studied vectors for transposition is that of AAV. One such study was that performed by Hodgetts *et al.* investigated CNTF, which was shown to promote survival and enhance long-distance regeneration of injured axons in the spine and brains of adults (Table 1) [14]. They utilized CNTF to test motor-related regions of the CNS to promote plasticity and regrowth of axons. Transduced with AAV1, the treatment, AAV-CNTF, coupled with mCHERRY (fluorescent tracking protein), was found to yield functional improvement over the control of AAV-GTP [14]. In another study, AAV was compared with umbilical cord blood cell-mediated (UCB-MC) therapy [10]. They found that cell-mediated (via UCB-MC) and direct GDNF (via adenoviral vector)

Table 3. Pros and cons table describing the overall status of gene therapies across spinal conditions.

| | Pros | Cons |
|---|---|---|
| Spinal cord injury | - Studies have revealed molecular alterations can lead to positive outcomes<br>- Several genes have been identified, with miRNA analysis showing positive potential | - Not many clinical studies on humans<br>- Some current studies lack statistical significance |
| Tumor | - Growing understanding of the molecular mechanisms in astrocytomas, ependymomas and hemangioblastomas, and meningiomas<br>- New variants and genetic targets are identified | - Studies are still very limited<br>- With multiple mechanisms involved in cancer, correcting one sequence can negatively impact another |
| Disc regeneration | - Due to intradiscal environment being harsh for cell survival, gene therapy can be a great treatment option<br>- mTORC1/RAPTOR identified as potentially beneficiary protein targets | - Gene therapy is not widely studied in this setting<br>- Still largely in the experimental stage |
| Scoliosis | - Multiple gene targets and mutations identified<br>- CRISPR/Cas9 and RNAi show great potential | Whether mutating these genes have adverse secondary effects is yet to be seen |
| Spinal muscular atrophy | Multiple types of therapies have been identified that enhance SMN2 protein production | - These therapies do not have long follow-up studies<br>- High cost of treatment |
| Spina bifida | - Genes have been identified for neural tube defects<br>- BMSC and CRMP4 siRNA therapy was shown to have positive outcomes | There is a need for greater preclinical longitudinal studies on large animals prior to human analysis |

BMSC: Bone marrow-derived mesenchymal stem cell; CRISPR: Clustered regularly interspaced short palindromic repeat; SMN: Survival motor neuron.

improved motor function in rats with SCI (Table 2) [10]. Compared with viral vector, UCB-MCs resulted in higher preservation of myelinated fibers in remote segments of spinal cord [10]. In both groups, exogenous GFP and GDNF expression were upregulated, demonstrating that regardless of delivery, GDNF induced increase in Schwann cells in injured rat spinal cords (Table 1) [10]. Overall, the study illustrated that using UCB-MC-mediated gene therapy, though dependent on the content, resulted in greater functional recovery [10].

These studies demonstrate that there are factors with various receptors and roles that can help mediate and/or aid in recovery from SCI. What is clear from the literature is that is it possible to expand the AAV vectors for the treatment of SCI where astrocytes play a significant pathological role. More studies need to be conducted to further clarify these roles, especially in humans as many of these studies have been conducted on rat lab models (Table 3). Taking these data from the lab to clinical practice remains a challenge.

## Tumor

Spinal cord tumors (SCTs) carry difficulties not only in their presentation, but also in their management. Primary SCTs are rare, accounting for only 2–4% of CNS tumors [15,16]. Metastatic disease can also present as spinal pathology. In contrast to its cranial counterparts, there is much less known about the genetics and characteristics of SCTs. In addition, increased evidence is showing that the aforementioned component of SCTs differs from similar tumors found intracranially [15,17]. Though these tumors are not often seen, they are associated with a high rate of morbidity [18].

With regards to primary SCTs, one must understand the common types associated with various locations. Intramedullary SCTs compromise approximately 5–10% of SCTs, and are the most common location for spinal tumors in children [16,18]. Astrocytomas, ependymomas and hemangioblastomas are the most common intramedullary lesions seen in adults [16–18]. Astrocytomas can be seen more frequently in patients with neurofibromatosis (NF) type 1 expression, while ependymomas are often found in patients with NF type 2 (NF2) [18]. Astrocytomas are highly linked to the proto-oncogene, *BRAF*, a serine-threonine protein kinase *BRAF* linked to gliomas in the CNS [18]. In grade 1 astrocytomas, two major mutations are noted in Figure 1 that cause constitutive activity of MAPK pathway (Figure 1 & Table 1) [16]. Grade II and III/IV mutations are also discussed in Figure 1, as grade III/IV mutations specifically show the H3F3A K27M variant being expressed in malignant tumors of the midline, including the spinal cord [16,17]. Another common molecular indicator of an astrocytoma is mutated CDKN2A, which codes for the p16 tumor suppressor and mutated tumor suppressor p53, which was also found to be expressed in 80–90 % of spinal cord glioblastomas [16,17]. In addition, *p16* and *PTEN* mutations are also commonly noted in spinal astrocytomas [17].

Ependymomas are more commonly seen in adults, and the tumors noted in children and adults demonstrate clear entities [18]. Figure 1 describes the mutations present in ependymomas, as these mutations were examined and found in 100% of grade I spinal cord ependymomas and 50% of grade II (Figure 1) [18]. A noteworthy gene involved in ependymoma manifestation is the *NF2* gene, as approximately two-thirds of patients with NF2 will go on to develop SCTs [17]. In addition, spinal cord ependymomas demonstrate whole-chromosome anomalies,

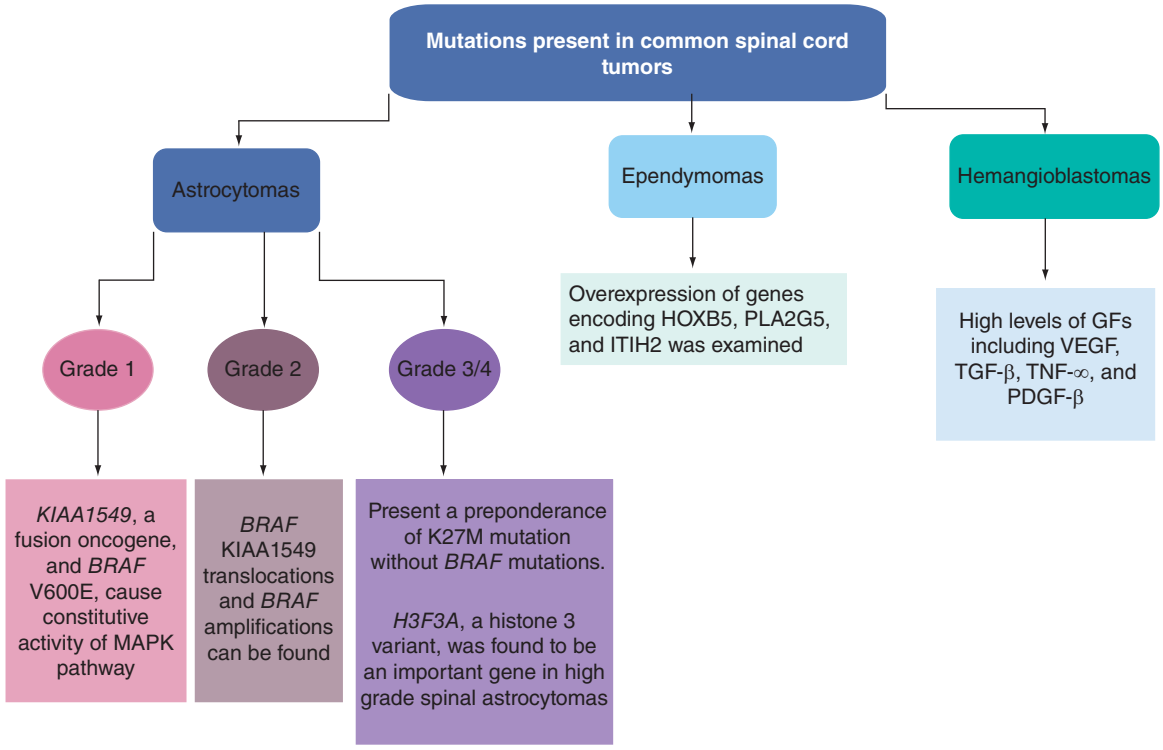


**Figure 1. The mutations present among astrocytomas, ependymomas, and hemangioblastomas spinal tumors.** Each of these mutations present a valuable target for gene therapy administration to restore normal function and cell-cycle control.
GFs: Growth factors.

**Table 4. Summary of the molecular indicators of subependymomas and myxopapillary ependymomas, two subtypes of ependymoma tumors.**

| Molecular indications of subependymomas | Molecular indication of myxopapillary ependymomas |
|---|---|
| Partial or complete loss of chromosome 6 | Chromosomal instability |
| Presentation of TCP1, ADM1 and Cdk11 | Overexpression of *NEFL* gene |

mutations affecting cliogenesis, microtubule assembly, and mitochondrial and oxidative metabolic pathways [18]. The expression of ependymoma subtypes are shown in Table 4 [17].

Hemangioblastomas are benign vascular lesions, with 20–40% of patients having evidence of von Hippel-Lindau (VHL) disease [16–18]. Spinal cord hemangioblastomas were noted to be strongly associated with VHL, occurring less frequently in sporadic cases [17]. The mutations of VHL mutants as linked to hemangioblastomas are shown in Figure 1 [16,18].

Extramedullary, intradural tumors include meningiomas. These types of tumors account for 25–38% of spinal tumors and commonly seen in adults [16–18]. Box 1 shows some of the characterized chromosomal, proteomic and genomic outcomes of meningiomas. Among the mentioned, NF2 is often presented in meningiomas. However, in cases of familiar spinal meningiomas that did not exhibit NF2, SMARCE1 was exhibited, and was associated with formation of multiple spinal meningiomas [16,17]. Matrix metalloproteins were also noteworthy, along with certain genes involved in intracellular and extracellular signaling (Box 1). Many studies have been conducted looking at intracranial meningiomas and gene targets for clinical treatments including bevacizumab, but more is needed to investigate these genes, and others in spinal meningiomas [16,17].

The BRAF protein has been targeted in some cancers, and based on its involvement in SCTs it also proves to be a valuable target for gene therapy [17]. BRAF has been targeted by utilizing hyaluronan, in addition to *Salmonella typhimurium* to suppress *in vivo* growth of spinal astrocytoma models [17]. Clinical trials are underway for patients

**Box 1. Molecular indications of extramedullary meningiomas.**

**Deletion of chromosome 22q**
Chromosomal losses of loss of 1p, 9p and 10q
Chromosomal gains of 5p and 17q
Presentation of NF2
MMP-9 upregulated
MMP-1 and MMP-2
*Hox* genes and *NR4* family
Genes in intracellular signaling (*RGS16, DUSP5,* etc.)
Genes in extracellular signaling (*TGFB114, IL-1B,* etc.)

These include chromosomal anomalies along with certain protein and gene expression.
NF2: Neurofibromatosis type 2.

with NF2 and meningiomas, including everolimus, PDGFR inhibitors and AR-42 [18]. Gene therapy involving suicide genes have been of particular interest in aiding in SCTs therapy [19]. In one study, plasmid-encoding herpes simplex virus-thymidine kinase and ganciclovir (GCV) was investigated using cationic, amphiphilic copolymer and PgP as a gene carrier (Table 1) [19]. Utilizing rat-induced SCT models, the study demonstrated the efficacy of PgP as a carrier and efficiently delivered reporter genes [19]. When comparing PgP/plasmid-encoding herpes simplex virus-thymidine kinase and GCV to carrier/suicide gene alone, GCV demonstrated significantly higher anticancer activity and also increased the suicide effect of cells, as well as apoptosis of tumor cells [19]. Additionally, it was shown to reduce the tumor size in the SCTs rat models [19]. Overall, the literature demonstrates vast information in regards to identified genes, targets and treatments in intracranial tumors, but has limited studies involving gene therapy of SCTs (Table 3). Hence, more information and studies need to be focused on targeting specific genes noted in SCTs.

## Disc regeneration

Degenerative disc disease (DDD) is a biomechanically related continuum of molecular, biochemical, cellular and anatomic alterations evolving over time that most often lead to chronic neck and back pain [20,21]. Degenerative changes in the intervertebral disc (IVD) cause loss of normal spine structure and function that is associated with the breakdown of the extracellular matrix (ECM) such as proteoglycan and type 2 collagen, decreased IVD height and inflammation [20,22,23]. Standard medical treatment very often only provides a short-term solution and in extreme cases, surgery may be warranted which can result in function loss, immobilization and potential additional complications due to the altered biomechanics [20,21]. Effective long-term treatments have therefore remained elusive as it has been proven difficult to reverse, halt or even delay the degenerative process due to the limited regenerative potential of IVD tissues [21,22].

Over the past 20 years, there has been a shift toward more promising therapeutic approaches for DDD that have now reached third-generation biopharmaceuticals. These biological therapies for IVD degeneration can be divided into three major groups: growth factor injection with or without a carrier, cell-based therapy with or without a scaffold, and gene therapy modifying endogenous gene expression and function which aids to restore or maintain the ECM [21,22,24]. Gene therapies may be used to produce products that block catabolism in degenerated IVD, enhance anabolism or reverse the degenerated disc state; thus, improving the catabolic and anabolic balance [20,22]. Because the IVD is avascular and encapsulated, and the intradiscal environment is harsh for cells to survive, local administration and direct delivery gene therapy is the best route. Taking into consideration that once a therapeutic gene is successfully transferred into the target cells, these genetically modified cells continue to produce the desired gene products thus making disc degeneration and related chronic conditions good candidates for such therapies [21].

Gene therapy delivery systems for IVD degeneration can be conducted using both *in vitro* and *in vivo* viral and nonviral vectors. The most commonly used viral vectors are AAV and lentivirus. Furthermore, RNAi and CRISPR/Cas9 are new technologies that have further enhanced viral vectors. Conversely, liposomes, polyplex micelles and exosomes are examples of the classic nonviral vectors utilized in IVD regeneration [21,25].

RNAi has been developed for downregulating harmful gene expression in the degenerated disc, leading to decelerated disc degeneration. Furthermore, the mTOR signaling as a target of gene therapy is an important emergence in this particular field. The mTOR plays a negative role in autophagy by regulating autophagy-related

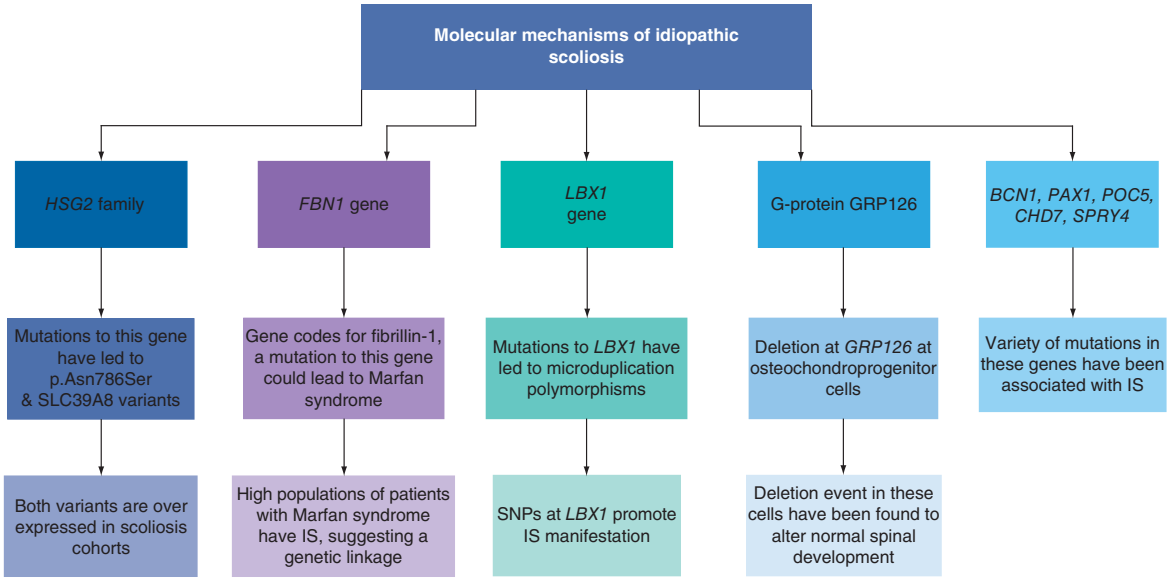


**Figure 2. An overview of the many molecular targets of idiopathic scoliosis and how they are linked to the diagnosis.** Each mutated target presents possibilities for gene-therapy administration.
IS: Idiopathic scoliosis.

proteins and lysosome biosynthesis (Table 1). A recent update published by Takeoka *et al.* proposes selective interference of mTORC1/regulatory-associated protein of mTOR to protect disc cells from inflammation-induced apoptosis, senescence and prevent ECM catabolism [11]. Furthermore, Farhang *et al.* demonstrated the use of lentiviral CRISPR epigenome editing systems as they were introduced into human degenerative disc cells to downregulate TNF receptor 1/IL-1 receptor 1 expression (Table 1) [26]. The results displayed the efficacy and feasibility of CRISPR-Cas9 system in pathological disc cells and also revealed a limitation in epigenome targeting of IL-1 receptor 1, which indicated that a tailored approach may be required for successful regulation of each gene [21,23].

Gene therapy development for DDD is promising, but still largely at the clinical trial and/or experimental stages with significant potential for clinical development (Table 3). Finding the best targets for gene therapy as well as addressing safety aspects, transfection efficacy and high costs are some of the major obstacles and limitations that if overcame, could lead to a breakthrough in disc regeneration research in the upcoming years.

## Scoliosis

Scoliosis is another spinal pathology that could be used as a possible target for gene therapies. Scoliosis is trademarked by a curvature in the vertebrae that causes asymmetry of the spinal cord. The common form of scoliosis is known as idiopathic scoliosis (IS), with its underlying cause being uncharacterized. As the causes of IS continue to be investigated, researchers have found a genetic link in its manifestation [27,28]. The curvature of the spine is a common human attribute that has many genetic components, with some genetic abnormalities or chromosomal disorders being found to have an ultimate cause of scoliosis. To date, there are many genes that have been suspected of causing scoliosis, but the familiar form of the disease has yet to be characterized. Moreover, the hereditary pattern of scoliosis has yet to be understood [29].

Through exome sequencing, some candidate risk genes have been identified as potential causes of scoliosis. This includes a variant of the *HSPG2* family known as *p.Asn786Ser*, a missense variant of *HSPG2* genes that has been reported to be overexpressed in cohorts of individuals with scoliosis (Figure 2) [20,25]. Another missense variant of *HSPG2*, *SLC39A8*, has been reported to cause spinal cord curvature, highlighting this locus for significance (Table 1). Also, some evidence reveals that the gene, *FBN1*, is involved in the formation of IS [20,30]. *FBN1* codes for the fibrillin-1, which is a key protein involved in providing structural support to elastic and inelastic connective tissue in the body (Figure 2) [31]. Its expression has been found to be linked to individuals with Marfan syndrome, where high proportions of patients with this disease develop scoliosis [20]. Moreover, other genome-wide assocation

studies (GWAS) have identified more genes that are associated with IS, such as *LBX1* [21]. The function of *LBX1* is yet to be fully characterized; however, it has been found in neuronal tissue, notably on spinal neurons and the hindbrain, indicating further neural potential [32]. With microduplication events at a specific region of chromosome 10q24.31, wild-type function of *LBX1* may be compromised in a way that manifests into scoliosis (Figure 2) [20,23]. GWAS studies have also identified the G-protein, GPR126, to have a possible linkage to scoliosis [20]. A deletion in *GPR126* at osteochondroprogenitor cells has been seen to alter expression of cartilage and normal spinal column development (Figure 2) [20]. Osteochondroprogenitor cells are specific cell types that can develop into either cartilage or bone through proper signaling events. This makes a deletion in these cell types significant in normal spinal development. At last, other genes such as *BCN2* have been found to be overexpressed scoliotic phenotypes, while specific SNPs of variants near *SOX9* and *KCNJ2* have also been linked to pathologies that manifest into spinal curvatures (Andersen-Tawil syndrome and campomelic dysplasia) [20]. Other genes that may also have association with scoliosis include: *PAX1, POC5, CHD7* and *SPRY4*. While the exact characterization and mechanisms of these genes may not be fully identified, their association with IS present them as potential targets for gene therapy (Figure 2) [20,22,24,33].

Scientists and physicians are looking to use gene therapy to develop noninvasive spinal fusion techniques that could replace spinal fusion surgery or minimally invasive scoliosis surgery. However, due to scoliosis mutations having lots of polymorphisms and variants, scoliosis genetic therapy is often focused on reducing the effects of these variants [34]. As mentioned with disc regeneration, one method of minimizing these effects is through CRISPR/Cas9 [13]. CRISPR/Cas9 has been investigated to repair *HSPG2*'s SNP, *SLC39A8*, in order to knock out its adverse spinal function by silencing or repairing the mutated sequence [12]. A research team, based at Washington University, MO, USA, performed an exome-wide association study of 457 severe adolescent IS cases where they identified the *SLC39A8* gene being prevalent [12]. In addition, with the *SLC39A8* gene being known to code for the upregulation of manganese cofactors, these researchers used CRISPR to reduce manganese intake. When the researchers used CRISPR/Cas9 to reduce manganese influx mediated by the *SLC39A8* on zebrafish, they found prevalent growth impairments in the spine (Table 1). This suggests that reduced manganese may be a factor in scoliosis development [12]. This also outlines the potential usage of CRISPR/Cas9 to be studied at the same locus to cause a gain-of-function of the membrane transport of manganese, should the right sequence be introduced. In addition, another gene, *PPP2R3B*, has also been studied with CRISPR/Cas9 in zebrafish [13]. *PPP2R3B* is linked to Turner's syndrome which is further linked to IS. A frameshift mutation at this particular gene was caused using CRISPR/Cas9, resulting in a scoliotic phenotype that was very similar to the human condition [13]. The defects seen included reduced mineralization of vertebrae that had resemblance to conditions like osteoporosis [13]. Although it has yet to be studied in skeletal tissue maintenance, this study indicates that *PPP2R3B* can be potentially silenced and eliminate IS in Turner's syndrome [13]. Moreover, CRISPR/Cas9 can be theoretically studied on other SNPs, such as those specific to the *LBX1* that upregulate the expression of adolescent IS. *LBX1* has been seen to be problematic when overexpressed as it leads to the genetic chain reaction that evidently leads to scoliosis [35]. Therefore CRISPR/Cas9 activity can be used to knockout this overexpression and silence *LBX1*. Moreover, this could also be done using RNAi. As previously mentioned, RNAi is a natural process of gene silencing that regulates expression and promotes knockout effects of certain genes through the activity of RNA induced silencing complex. RNAi was done on *FBN1* in *Drosophila*, which silenced the frataxin protein and ultimately led to the development of Friedreich's ataxia and complications such as scoliosis (Table 1) [36]. *BCN2* can also be used as a valuable target in RNAi, as its overexpression can be silenced using RNAi in order to downregulate its adverse effects on spinal development.

With the exact genetic components to scoliosis yet to be fully characterized, it could be problematic using gene therapy for treatment of scoliosis without knowing the subsequent effects that mutating candidate risk genes can have. Scoliosis is a polygenetic disease, meaning that it can be affected by multiple genes, complicating its usage in gene therapy. This is a limitation on gene therapy usage for IS, as the collection of its genes are yet to be fully characterized (Table 3). This means using CRISPR/Cas9 or RNAi at a specific sequence may or may not eliminate the phenotype. In addition, by altering a certain sequence, or by silencing it all together, it can lead to implications where a new phenotype can be produced that may or may not be desired. In addition, another major limitation about IS gene therapy is that there is yet to be a reliable way to predict the progression of the phenotype [20]. IS has been analyzed as autosomal dominant; however, due to IS being more prevalent in females, a sex-linked component to its expression may be indicated. However, despite these limitations, with further GWAS and sequencing analysis, the molecular mechanisms that manifest into scoliosis can be further characterized for future clinical practice.

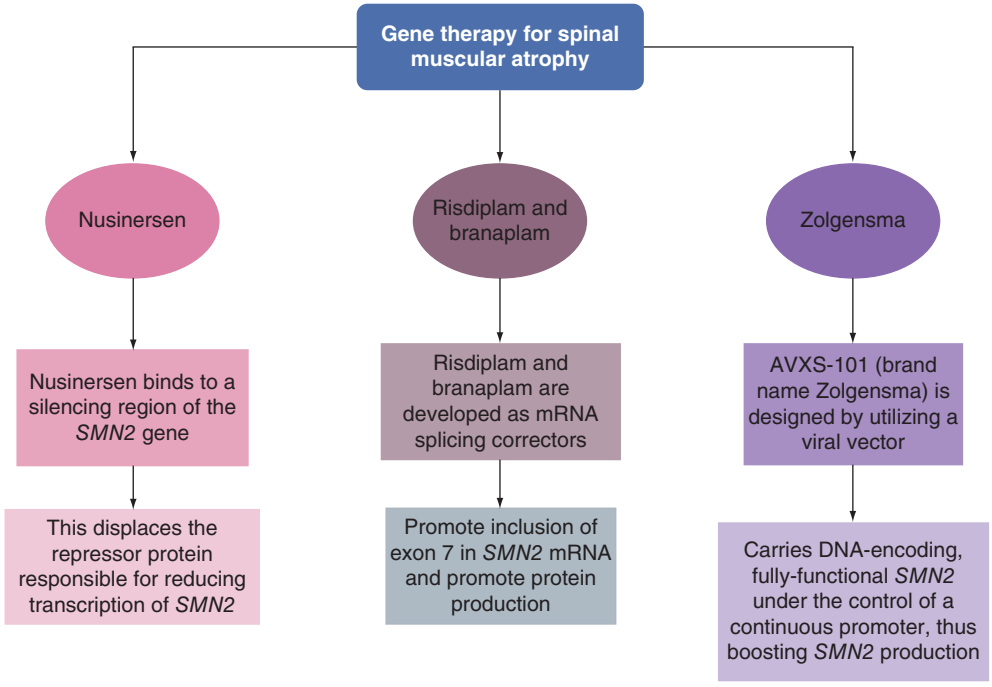


**Figure 3. Examples of current genetic therapies studied for spinal muscular atrophy.** Each technique seeks to restore optimal production of the SMN2 protein, which is compromised during SMA expression.

## Spinal muscular atrophy

SMA is a neurological disorder that causes progressive degeneration of lower motor neurons. It is most commonly classified into three types, with type I SMA being the most severe and typically causing death before 2 years of age [37]. Newer gene therapies can not only slow the progression of this disorder, but possibly even ameliorate the damage it causes. In 95% of SMA cases, the gene expressing the SMN1 protein, which is crucial for motor neuron development, is homozygously deleted from chromosome 5q [37]. However, SMN1 is not the only protein that is pivotal for proper motor neuron development. In fact, another protein named SMN2 has also been discovered to play an important role in normal development. Researchers have found that the degree of expression of SMN2 correlates directly with length of survival and improved motor development in individuals with SMA [37]. Thus, several experimental gene therapies have been focused on increasing expression of SMN2 as much as possible.

At the forefront of therapies for SMA is nusinersen®, an antisense oligonucleotide. Figure 3 describes the function of nusinersen on SMN2 repressors in more detail. Nusinersen is administered intrathecally to bypass the blood–brain barrier and has had very promising success in several randomized, placebo-controlled, double-blinded clinical trials (Table 1). Populations of SMA patients treated with nusinersen have shown objective and statistically significant improvements in neurological function, remarkable motor milestone achievements and prolonged survival without the need for permanent ventilation [38–40]. These beneficial results were seen in patients of different ages with various types of SMA, with adverse effects proving to be minimal. These were limited to mild elevations in urinary protein and thrombocytopenia, both of which were not specific to the treatment group, as well as complications with intrathecal administration that were not different from what can be expected with any procedure involving a lumbar puncture. As the first promising therapy to improve patient outcomes and survival in patients with SMA, nusinersen gained approval by the US FDA in December 2016 for use in patients with any form of SMA [41].

With the advent of nusinersen, various other genetic therapies were subsequently developed and have shown efficacy in treating SMA, including risdiplam® and branaplam®. Figure 3 describes the mechanism they follow to enhance SMN2 (Figure 3 & Table 1) [37]. Risdiplam has been evaluated in two Phase II/III trials named FIREFISH (open label) and SUNFISH (placebo controlled) assessing its effectiveness in treating SMA1 patients between 1 and 7-months-old and SMA2/SMA3 patients aged between 2 and 25-years-old, respectively [42,43]. Both trials demonstrated improvements in motor function correlated with increased blood levels of SMN protein, while also

being well tolerated by the participants with no reported major adverse effects. On the other hand, studies with branaplam are ongoing after an initial study was suspended due to demonstrated nerve injury in a preclinical toxicology study [44]. AVXS-101 (brand name Zolgensma) is another innovative gene therapy (Figure 3). It utilizes a vector designed and its molecular effects are summarized in Figure 3 [37]. Mendell *et al.* carried out the first single-center, open-label trial that tested both safety and efficacy of a single intravenous dose of AVXS-101 in 15 children with SMA1 and two copies of *SMN2* [45]. All patients that were treated reached at least 20 months of age without the need for permanent ventilation, achieved new motor milestones never seen in prior studies (such as crawl, pull to stand, stand, independent walk and speaking) and continued improvement in these findings observed at the 24-month follow-up time point. Adverse effects were minimal and largely unrelated to treatment. This groundbreaking success prompted FDA approval of Zolgensma in May of 2019 for the treatment of SMA in children <2 years of age with bi-allelic mutations in the *SMN1* gene (Table 1) [46]. Several additional multicenter trials are ongoing to build on these initial findings.

Overall, these genetic therapies for SMA are expanding therapeutic options for patients with this once inevitably fatal disease. Although high costs remain a significant barrier to the widespread adoption of these therapies (e.g., one dose of nusinersen costs US $125,000, with treatment regimens calling for four doses in the first 2 months of treatment and one dose every 4 months after) (Table 3). Costs to the patients may reduce over time as the success of these gene therapies grows, more efficient production strategies are put in place, and subsidization becomes more readily available [37].

## Embryologic spine conditions

Embryological errors in development of the spine are most often related to failure of neural tube closure, most commonly resulting in a condition known as spina bifida. Spina bifida results from failure of the fusion or development of part of the vertebral arch in utero, and the extent of this nonfusion influences the neural development of the embryo [47]. Patients typically require surgery either in utero or at birth to repair the spinal defect and manage other associated comorbidities. Various environmental such as folic acid deficiency can cause spina bifida; however, the genetic component involved in its development is estimated at 60–70%, as research has shown that families often have multiple cases [48]. While researchers have not been able to identify many genes that are related to neural tube defects, they have observed how mutation in variants the related to planar cell polarity pathway, folate metabolism and the glycine-cleavage pathway can have a role [48]. For example, when Narisawa *et al.* generated mice with a knockout of key enzymes in the glycine cleavage system pathway, they observed a high incidence of neural tube defects not observed in the wild-type mice [49]. Although experiments like this may shed light on specific genetic components involved in the human development of neural tube defects, researchers are actively working to identify genomic and epigenetic consistencies among patients with neural tube defects that may be targeted with innovative gene therapies.

Current research has revealed growing promise in the utilization of combined stem cell and genetic therapy delivered in utero for the treatment of neural tube defects identified via ultrasound imaging. A preclinical rodent study completed by Wei *et al.* evaluated the efficacy of combined bone marrow-derived mesenchymal stem cell (BMSC) therapy and siRNA of CRMP4 delivered together intra-amniotically in rat fetuses with neural tube defects [50]. CRMP4 was identified as a protein that was significantly upregulated in rat embryos with neural tube defects, and thus was targeted with siRNA to reduce its expression with the hopes of treating affected rat fetuses. These researchers observed that combined BMSC and CRMP4 siRNA therapy was shown to repair skin lesions surrounding the neural tube defect, improve motor neural function (assessed by EMG), reduce neural apoptosis, and promote expression of neural differentiation-related molecules and neurotrophic factors in the spinal cord of rat fetuses with spina bifida (Table 5) [50]. The results of this experiment suggest that in utero delivery of CRMP4 siRNA combined with BMSC could potentially treat neural tube defects in humans. Other rodent studies illustrated similar successes. For example, adenovirus-mediated intra-amniotic injection of genes for BDNF in rats with spina bifida showed increased BDNF around the lesion, reduction of pro-apoptotic cells, upregulation of anti-apoptotic cells and increased neurogenesis in the dorsal-root ganglia of the spinal cord (Table 5) [51]. Although these preclinical results demonstrate the theoretical feasibility as well as objective molecular and clinical improvements brought about by gene therapy used to treat neural tube defects, there is still need for further preclinical studies with large animals and longitudinal analyses of effectiveness before understanding its true potential in humans.

Table 5. Major findings of studies analyzing spinal bifida for its gene therapy potential.

| Study (year) | Major finding | Ref. |
|---|---|---|
| Wei *et al.* (2020) | -CRMP4 is significantly upregulated in rats with neural tube defects<br>- CRMP4 and siRNA therapy reduced neural apoptosis and promoted neural differentiation in the spinal cord of rat fetuses with spina bifida | [50] |
| Pedram *et al.* (2017) | AAV-mediated injection with BDNF resulted in less pro-apoptotic cells and more anti-apoptotic cells, and increased neurogenesis at spinal cord | [52] |
| Narisawa *et al.* (2012) | A knockout of key enzymes in the glycine cleavage system pathway resulted in high incidence of neural tube deficits | [49] |

AAV: Adeno-associated virus.

## Conclusion

Through greater success in investigating the genetic properties of various spinal pathologies, advancements in robotic technology have led to promising biomedical developments. The future of gene therapy could present opportunities for even greater efficiency through the usage of small-scale robotics and intelligent carriers for gene delivery. The biomedical development of small-scale robotics to target gene sequences has led to the potential of greater precision in targeting genes, transferring desired oligonucleotide sequences and eliminating pitfalls in conventional gene therapy such as delivery control, manipulation concerns and possible reduced therapeutic efficiency [52]. The development and application of nanotechnology such as nanobots and microrobotic systems have been characterized to provide safer and more precise methods in gene delivery than viral vectors [52]. This not only outlines the future of biomedical developments, but could allow for greater success in the usage of gene therapy in treating spinal pathologies.

## Future perspective

As gene therapy continues to be investigated as a treatment modality, its outcomes will be expected to have profound effects in the future of clinical care. To date, much of the expectation within the healthcare community is that gene therapy will be regularly used in the next 10–20 years to ameliorate and even cure devastating pathologies. This expectation is based on the expansion of our understanding of the human genome, our analysis of mutation sequences and identification of key genetic targets that could contribute to diseases and deformities. This is significant as most spinal pathologies are polygenic, meaning that as more gene sequences are identified, more targets and receptors for gene therapy administration can be discovered. Moreover, as further research continues, gene therapy will become more refined with improved viral vectors, improved genomic analysis, along with the development of improved gene-therapy models and techniques. At the moment, gene therapy is developing slowly, and is not expected to be introduced into mainstream clinical practice in the near future. However, the number and variety of studies involving gene therapy is advancing and will continue to be studied for academic, and potentially therapeutic, purposes. The current outcomes are encouraging, and while gene therapy does have its current limitations, it is expected to develop into one of the most revolutionary healthcare staples of the 21st century.

### Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

No writing assistance was utilized in the production of this manuscript.

## Executive summary

**Gene delivery**
- In terms of the delivery mechanism, gene delivery remains a challenge in the field as it is highly dependent on the vector, the desired location of treatment and minimization of side effects.
- The majority of spinal disorders are ideal for gene therapy because they are localized and monogenetic.

**Spinal cord injury**
- Neurotrophic factor delivery to the epicenter of spinal cord injury demonstrated response with enhanced neuronal survival and axon growth.

**Spinal cord tumors**
- Gene therapy involving suicide genes have been of particular interest in aiding in spinal cord tumor therapy.

**Degenerative regeneration**
- RNAi has been developed for downregulating harmful gene expression in the degenerated disc, leading to decelerated disc degeneration.

**Scoliosis**
- Scoliosis is a polygenetic disease, meaning that it can be affected by multiple genes, complicating its usage in gene therapy. This means using CRISPR/Cas9 or RNAi at a specific sequence may or may not eliminate the phenotype.

**Spinal muscular atrophy**
- As the first promising therapy to improve patient outcomes and survival in patients with spinal muscular atrophy, nusinersen gained approval by the US FDA in December 2016 for use in patients with any form of spinal muscular atrophy.

**Embyologic spine conditions**
- Current research has revealed growing promise in the utilization of combined stem cell and genetic therapy delivered in utero for the treatment of neural tube defects identified via ultrasound imaging.

**Future perspective**
- As further research continues, gene therapy will become more refined with improved viral vectors, improved genomic analysis, along with the development of improved gene-therapy models and techniques.

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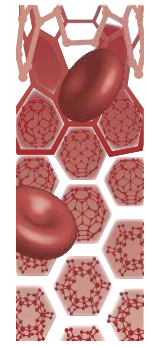

# Virus-mimicking nanocarriers for the intracellular delivery of therapeutic biomolecules

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"nanoparticles with viral proteins or virus-like structures, may hold promise as intracellular delivery tools for therapeutic biomolecules in the future."

First draft submitted: 24 February 2020; Accepted for publication: 28 February 2020; Published online: 15 April 2020

**Keywords:** drug delivery • gene therapy • nanoparticles • viral vector • virus-like particles

## Current challenges for therapeutic biomolecules

Biomolecules are of interest for the therapy of diverse diseases. Modalities of therapeutic drugs that utilize biomolecules include hormones, peptides, antibodies, DNA, RNA and ribonucleoprotein complexes. Among them, antibodies have been widely utilized for the treatment of various diseases [1]. Antibodies usually target extracellular molecules, such as soluble factors or cell surface antigens, followed by the inactivation of target molecules or other modes of action. While the antibody-based therapeutics against extracellular targets are successful, antibodies are still unable to target intracellular molecules, due to their inability to penetrate cellular membranes because of their large size (>150 kDa) [2]. The common problem with therapeutic biomolecules, such as proteins, DNA or RNA, is their large size compared with conventional small molecule drugs. This makes it difficult to penetrate cell membranes and causes issues for intracellular delivery, which is the key to therapeutic efficacy of biomolecules [3]. Moreover, current promising modalities utilizing biomolecules, such as siRNA or genome editing tools including Cas9-gRNA complex, as well as immunogenic *in vivo* are potentially unstable [4]. Therefore, these therapeutic biomolecules must be protected from degradation and the host's immune system.

## Synthetic nanoparticles: achievements & challenges

Nanoparticles have attracted academics and pharmaceutical industries because of their efficient delivery of therapeutic drugs [5]. Patisiran (Onpattro®) is a good example of a current and successful nanoparticulate-delivery system for nucleic acids. Patisiran is the first US FDA-approved drug utilizing RNAi for therapeutic silencing of a target gene. Patisiran targets the transthyretin gene, whose mutant form can cause hereditary transthyretin-mediated amyloidosis (hATTR) [6]. Patisiran consists of two components: a lipid nanoparticle (LNP), acting as the carrier, and an siRNA, serving as the active pharmaceutical ingredient [7]. LNPs are approximately 50 nm in diameter and contain ionizable lipids. After intravenous infusion, LNPs are coated with apolipoprotein E in the blood, followed by low-density lipoprotein receptor-mediated endocytosis into hepatocytes. After endocytosis, ionizable lipids become positively charged in a low pH environment in endo/lysosomes, and then fuse with the endo/lysosomal membrane, followed by the release of siRNA into the cytoplasm and the induction of RNAi [7].

Although a clinical success, the LNP platform still has disadvantages including no targeting ability except for liver, toxicity and low endosomal escape efficiency [7]. Several studies revealed that LNPs only achieve a low endosomal escape efficiency of payload siRNA (1.0–3.5%) [8,9]. Despite two decades of efforts since the first development of an LNP prototype [10], we have achieved little progress in the intracellular delivery of biomolecules. Currently, another concern has arisen in the long-believed, enhanced permeability and retention (EPR) theory of nanoparticle-based drug delivery. The EPR effect is the theory that large molecules or nanoparticles can passively accumulate in a tumor. This is probably due to the heterogeneity of tumor tissue in patients, as the EPR effect has not always been

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successful in patients, and is often observed in experimental mouse tumor models [11]. Under these circumstances, there is an immediate need to overcome the challenges that limit the clinical application of current synthetic nanoparticles.

## Viral vectors for delivery of biomolecules

Apart from synthetic nanoparticles, viral vectors are other options for the delivery of biomolecules, especially DNA and RNA. To date, several viral vectors have been developed, with adeno-associated virus (AAV) being the most extensively studied DNA delivery platform in gene therapy [12]. Since AAV has been known as a nonpathogenic human virus, it is relatively safe compared with other viral vectors. From the first approval of the AAV-based therapeutic drug by Glybera® (alipogene tiparvovec) in Europe in 2012, to the recent approval of Zolgensma® (onasemnogene abeparvovec) by FDA in 2019, it is now clear that AAV is a promising platform for gene therapy [12].

Although, numerous clinical trials using AAV as a DNA vector are ongoing, some drawbacks should be addressed in the current AAV platform, such as anti-AAV immunity in certain populations, low capacity of payload DNA (usually limited to ~4.5 kilobases) and potential integration at unwanted sites in the host genome [13,14]. While engineering of AAV vectors potentially overcomes a few of these drawbacks [12], AAV vectors should be modified to become a versatile platform.

## Synthetic nanocarriers with viral functions for the delivery of biomolecules

Besides synthetic nanocarriers and viral vectors, the synthetic nanocarriers mimicking viral function, for instance, virus-like particles (VLPs) offer an appealing platform for the delivery of biomolecules. VLPs are composed of the viral proteins with or without lipid envelope and resemble the surface structure of parental viruses. VLPs have long been utilized as an immunogen of prophylaxis vaccines, such as human papillomavirus vaccine and hepatitis B virus (HBV) vaccine. Since VLPs contain no genetic material of parental viruses and can display viral proteins that are involved in infection machinery, biomolecules incorporated inside VLPs can be delivered to target cells that are susceptible to parental viruses [15]. Specifically, viruses can penetrate cellular membranes by membrane fusion or a disruption mechanism, by utilizing viral surface proteins [16]. Therefore, VLPs can achieve efficient intracellular delivery of payload biomolecules.

For example, VLPs displaying the vesicular stomatitis virus envelope G protein (VSV-G) can deliver the Cas9-gRNA complex and achieve genome editing *in vivo* [17]. Because of a strong fusogenic activity of VSV-G, the VLPs can penetrate cellular membranes and deliver functional Cas9-gRNA complexes into cells. This system enables traceless delivery of genome editing tools without using any genetic material, leading to reduced off-target effects. Other applications of VLPs include, siRNA delivery using JC polyoma virus-like particles [18] and mRNA delivery using alphavirus-like particles containing core/envelope proteins [19]. In addition to VLPs, a viral capsid-like structure (i.e., protein nanocage) formed by *in silico*-designed proteins, has been utilized to encapsulate its own RNA [20]. Using this approach, the functionality of protein nanocages can be further designed *in silico* to improve its delivery efficiency.

Our group has been focusing on drug delivery using VLPs, especially HBV-like particles (bio-nanocapsules [BNCs]) [21]. Since envelope proteins of HBV have pleiotropic functions, such as receptor recognition [22] and membrane fusion [23], BNCs can deliver their payloads into hepatocytes like HBV. As a practical application of BNCs, it was recently demonstrated that anti-CD11c antibody-conjugated BNCs can deliver proteinous antigens into dendritic cells and elicit strong immunity in mice [24].

Although these VLPs mimicking viral functions are promising, some drawbacks should be taken into account such as, no efficient drug loading method, immunogenicity due to viral proteins, possible *in vivo* toxicity and scalability of mass manufacturing. Engineering VLPs could potentially solve issues discussed previously, such as lowering immunogenicity of VLPs by protein engineering [25].

## Conclusion

Current synthetic nanoparticles and viral vectors have advantages and disadvantages. We discussed here that nanoparticles with viral proteins or virus-like structures, may hold promise as intracellular delivery tools for therapeutic biomolecules in the future. In addition to the development of nanomaterials for the delivery of biomolecules, it is crucial to understand how our body responds to these nanomaterials, in terms of antigenicity, biodistribution, clearance from the body, interaction between nanomaterials and cells and intracellular fate of nanomaterials.

Financial & competing interests disclosure

This work was supported by JSPS KAKENHI (Grant-in-Aid for Scientific Research [S] 16H06314 to S Kuroda; Grant-in-Aid for Early-Career Scientists 18K18386 to MS), Japan Agency for Medical Research and Development (AMED) (19fk0310105h0003 to SK), and the 'Dynamic Alliance for Open Innovation Bridging Human, Environment and Materials' (MEXT, Japan). The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

Writing assistance was utilized in the production of this manuscript by Editage (funded by SK, 16H06314).

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