AAV as a Vector for Muscular Dystrophy Gene Therapy

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Abstract There is a growing interest in gene therapy, which is due to improved understanding of new gene delivery vectors and active support by the pharma industry and the FDA. One of the delivery vectors is the Adeno-associated virus (AAV), which is a virus that can be used to deliver deoxyribonucleic acid (DNA) to a target cell. The use of engineered recombinant AAV (rAAV), which does not contain any viral genes, for enhanced specificity and as a tool for treating various diseases has thus far been proven to be one of the safest strategies for gene therapy. While significant advancements are made, there are still challenges considering effective delivery to target tissues and the aspects of safety, durability, and efficacy of the gene therapy.

Preface

On our search for an interesting topic in the field of genetic engineering and biotechnology we came across the subjects of “stem cell therapy”, “Bionic Limbs (mind-controlled prosthesis by the University of Michigan)” and “Organ-on-a-chip” technology; however, none of these interested us. There was already another group who had chosen to write about stem cell therapy, and it seemed that it would be difficult to find a researcher or representative for the interview concerning the other two topics. Through Loïc’s father we got contact and an affirmation for an interview with Dr. Judith Reinhard at the “Biozentrum” Basel. We asked her what she thought about our three topics and what she was currently researching. She answered that she was not working on any of these but would be willing to do an interview about her research on AAV-Based gene therapy. As we researched about her work, we became very intrigued by it, so we gladly chose it for our paper and accepted the offer for the interview.

It seems counterintuitive to use a virus to drive health, which is one reason why the subject instantly caught our interest. However, to be of use in healthcare, the virus of course must be optimized for the use of gene therapy. Immediately, the questions arose: “What components have to be optimized?”, “What are their effects?” and “what risks have to be considered?”

Introduction

The AAV vector

Adeno-association viral (AAV) vectors were invented in the mid-1980s. They are frequently used in a wide range of diseases, like Alzheimer’s, Parkinson’s, Duchenne and laminin-A2 muscle dystrophy. They are used to spread the medicine the vector is carrying and disperse it throughout the body. Furthermore, they have become very popular in clinical trials, because the AAV vectors carry DNA segments of interest for a variety of people while not carrying any viral genes. This all results in the AAV vector being the safest strategy for gene therapy. Intravenous therapy is used to disperse the vectors because it is an effective way to reach all affected parts of the body.

The AAV vector has become one of the leading gene delivery vectors in clinical development, but there are other options.

Non-viral vectors are a safe alternative to the AAV vector. Its lack of immunogenicity and cytotoxicity make it a much safer alternative that is low in costs and easy to produce. The one drawback it has is a major one: it is extremely inefficient in delivery, because the gene expression delivered by the vector is weak and short-lived.

Another alternative is the lentiviral vector. For the past two decades, lentiviral vectors, which stem from the human immunodeficiency virus, have been optimized and studied. The lentiviral vectors split the viral genome into separate plasmids to prevent reintegrated virus generation. Its need for at least three HIV-1 genes for production prevents it from being used in gene therapy because it is too dangerous, making the AAV vector the perfect alternative to it. The AAV vector-based gene therapy market is projected to become a 14.7-billion-dollar industry by 2030, which would mean it should almost triple.

Application

In the next section we will discuss a number of promising applications of the AAV vector.

Duchenne Muscle Dystrophy:

Duchenne Muscular dystrophy is an x-linked disease that kills 1 in 5000 males and affects them by the time they are 12 years old. Usually, they die in their thirties due to respiratory and cardiac issues. The disease is caused by the Duchene Muscular Dystrophy (DMD) gene that codes for dystrophin. Dystrophin is a structural protein that prevents damage-inducing muscle contractions; it protects the muscle fibres. The absence of this dystrophin causes the muscles to damage over time and compromises the stability of the muscle fibres. The only proven way to treat this illness is a gene transfer to restore dystrophin expression using AAV which is a non-pathogenic virus. So far, this method has yielded promising results when testing on large animals, but some tweaks and improvements are to be made to make it ready for use on humans.

Due to the large size of the DMD gene’s coding sequence, gene transfer therapies are difficult. The availability of the full-length sequence of dystrophin has allowed scientists to remove some coding

sequences in the gene, so researchers can create new genes that contain different versions of mini- or micro-dystrophins.

The AAV vector has been proven to work clinically when undergoing gene replacement therapy for spinal muscular atrophy. Several researchers in the United States of America are performing trials where they use AAV vectors to deliver these micro- or mini dystrophins.

The most promising results are achieved in Pfizer and Sarepta Therapeutics' clinical trials. Even though there is encouraging data, there are still issues to be solved such as full optimization of the dosage, immune control, as well as manufacture and supply challenges.

Lama2 Muscular Dystrophy:

In LAMA2 muscular dystrophy, the muscle cells lack a stabilizing factor. Researchers have developed a promising gene therapy to replace the missing factor. LAMA stands for the gene that encodes the protein laminin-A2, which is defective in affected individuals. This protein normally acts as the "putty" between cells, that binds to muscle fibres and confers their stability. The question came up if it is possible to use components of the protein agrin to replace this "anchor". Researchers have now succeeded to smuggle an optimized agrin component along with another replacement protein to act as anchors. Experiments in mice have produced promising results. To administer the proteins, they use modified viruses, which act as vehicles for the blueprints of the replacement proteins.

Description

AAV as a vector for gene therapy

The discovery of DNA as the biomolecule of genetic inheritance and disease opened the possibility of future therapies which use the altering of mutant, damaged genes for the improvement of human health. AAV is a virus which can be engineered to do exactly that. It can be used to deliver a transgene to a target cell. While wild type AAV is dangerous to the human body, the engineered version, the rAAV (recombinant AAV) which contains a DNA sequence of interest instead of the viral genes, seems to be one of the safest strategies for gene therapy.

Wild type AAV is a protein shell which surrounds and protects a single stranded DNA genome of about 4.8 kilobases. It is part of the parvovirus family and is dependent on co-infection with other viruses to replicate. The single stranded genome contains three genes: Rep (Replication), Cap (Capsid) and the aap (assembly). These genes give rise to at least nine gene products through the use of three promoters, alterative translation sites and differential splicing. The coding sequences are flanked by ITRs (Inverted Terminal Repeats) which are necessary for genome replication and packaging. The rep gene encodes four proteins which are also required for viral genome replication and packaging, the cap gene gives rise to the viral capsid proteins which form the outer capsid shell which protects the viral genome and the aap gene encodes the AAP (Assembly-Activating Protein) which has a scaffolding function for capsid assembly. The AAP has this function in the AAV2 serotype. However, its function differs between different serotypes.

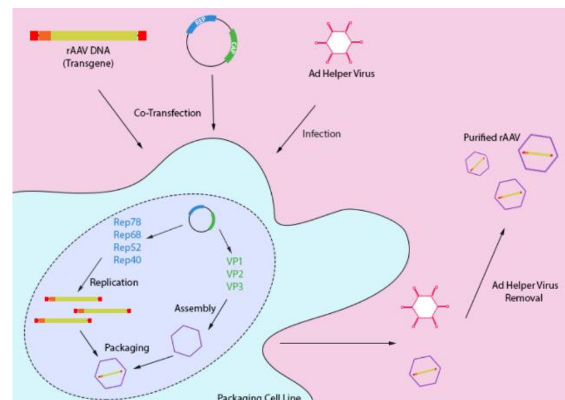

Fig. 1 Representation of rAAV production used here to show AAV assembly, important components and give an overview over its build

The only part left of the viral DNA are the ITRs. The ITR-flanked transgenes can form concatemers that persist as episomes in the nucleus of the transduced cells. There it doesn't integrate into the host's genome and will be diluted over time as the cell undergoes replication.

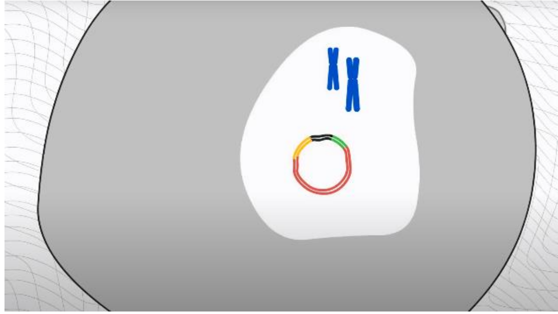

Fig. 2 Transgene persisting as an episome in the nucleus of the transduced cell, therefore not integrating into the cell's DNA

AAV Vector Designs

An important factor for the therapeutic use of AAVs is the packaging size. Trying to increase this over 5kb (including the ITRs) has been shown to decrease viral production yields or transgene recombination. An important consideration in the vector design relates to the biology of the single-stranded AAV-delivered transgenes. In the nucleus it must be converted into a double-stranded transgene, by synthesizing the missing strand, which takes time and is considered a limiting step in the onset of transgene expression. An alternative is to use self-complementary AAV, in which the single-stranded genome complements itself in the nucleus. However, this reduces the packaging capacity to 3.3 kb.

Even though many therapeutic strategies involve systematic vector delivery, it is often desirable to have cell- or tissue-specific expression to prevent leakage of the AAV particles to result in transduction and expression of the gene of interest in unwanted cells or tissues.

Sequences placed between the ITRs typically include a promoter, the gene of interest and a terminator. Strong, constitutively active promoters are used. An example is CMV (Cytomegalovirus) promoter. A problem is that in some cell types, certain promoters are silenced, which means that the repressor prevents the promoter from functioning. This must be considered. CMV promoter for example was shown to be silenced in the central nervous system. The choice of the used terminator also impacts gene expression. To support maximal tissue-specific expression, codon engineering should be performed.

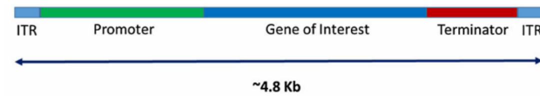

Fig. 3 Schematic representation of the gene insert transported by the rAAV

Although much is known about effects of certain components, it is not yet possible to predict how a particular design will function. Therefore, the final design needs to be determined empirically.

Selection and Optimization of the AAV Capsid

AAV has evolved to enter cells through interactions with carbohydrates present on the surface of target cells. Differences in sugar-binding preferences, encoded in capsid sequence differences, can influence cell-type transduction preferences of AAV variants. Based on this, there is a hypothesis that AAV may be able to pass the blood brain barrier (BBB) and infect cells of the central nervous system (CNS). In addition to primary carbohydrate interactions, secondary receptors have been identified that also play a role in viral transduction and cell and tissue selectivity of viral variants. Scientists have also inserted larger proteins into the capsid shell to confer selectivity.

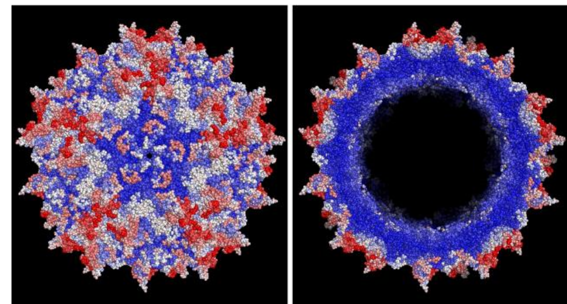

Fig. 4 AAV capsid

The choice of a particular AAV to use as a gene transfer vector is to a great degree reliant on which cell and tissue types are being targeted, the safety profile associated with the delivered gene, the choice of systematic or local delivery, and the use of tissue-specific or constitutively active promoters.

AAV Immunogenicity

AAV lacks engineered lipids or other chemical components that could contribute to unwanted toxicities or immunogenicities that may not be predictable or fully understood. AAV has been shown to be less immunogenic than other viruses. One reason is thought to be, as mentioned above, that rAAV does not contain any viral genes. Therefore, there will be no active viral gene expression to amplify the immune response. However, the capsid proteins and nucleic acid sequence delivered can trigger components of the immune system. Also, most people have already been exposed to AAV and have already developed an immune response to the variants they have been exposed to. The challenge is to deliver a therapeutically efficient dose of rAAV to a patient population, which already has an immunological memory against the virus. If the transgene is an engineered variant of such a virus, it can be recognized as foreign.

Pre-existing immunity to AAV is thought to be one of the factors in early clinical failures. Even though it can often be overcome by choosing an AAV variant which hasn't circulated in the human population yet, it is one of the main therapeutical challenges.

Delivery Strategies

The administration routes used in therapeutic use of AAV can be separated into two main groups.

In systematic administration, the AAV enters the circulatory system so that the entire body is affected. Systematic delivery is for example being used to target the liver. It is also used for intramuscular delivery. Once the skeletal muscle tissue has been transduced, the muscle cells serve as a production site for protein products that can act locally and systematically. Variants which have been shown to cross the BBB could be used for the treatment of diseases connected to the CNS and brain. Unlike local administration to the eye, which is considered an immune-privileged site, that might not be affected by pre-existing immune memory, systematic administration requires investigation for patient Nab (neutralizing antibodies) levels.

Local delivery is used to target a certain cell or tissue type.

Administration routes to the CNS range from direct intraparenchymal administration into areas of the brain, intracerebroventricular, and cisternal and lumbar intrathecal routes. Which route is the best depends on the disease and the affected areas of the brain. Also interesting is that the administration directly into the cerebrospinal fluid through an intrathecal route can result in wide CNS biodistribution, which is thought to be necessary for diseases such as spinal muscular atrophy (SMA). An alternative to that would be the already mentioned systematic administration of AAV variants which have been shown to cross the BBB.

A working strategy for the local delivery to the cardiac muscles has not been found yet, and it is still unsure why previous trials have failed.

Aerosolized AAV for inhaled pulmonary delivery was utilized in some of the earliest trials of cystic fibrosis (CF). None of these trials resulted in significant benefit or showed much of a response. Nonetheless, these efforts proved that AAV can safely deliver genes to the lung, which might be an ideal strategy for other diseases, such as influenza and other infection diseases of the lung.

Manufacturing

AAV needs to be produced in living systems.

The most used platform for rAAV production involves transfecting human embryonic kidney (HEK293) cells with three plasmids; one encoding the gene of interest, one carrying the AAV rep/cap genes, and another containing helper genes provided by either adeno or herpes viruses. While rates of about 10^14 GC/L (genome copies per litre) can be achieved, the lack of scalability is a significant limitation. Successfully delivering three plasmids to one cell is a relatively inefficient process.

Fig. 5 Incubator used for the controlled growing of cell systems at the Biozentrum Basel

In some platforms, some genetic compounds for the AAV manufacturing have been integrated into the genome of mammalian or insect production cell lines. Most viral helper genes needed for AAV production cannot be stably transfected, but there are exceptions. These exceptions include expression of the AAV rep gene, which is toxic to mammalian and insect cells. Because of this, two approaches have been used to develop mammalian cell lines. In the first, co-infection of baby hamster kidney (BHK) cells with two replication-defective HSVs (herpes simplex virus) which are engineered to encode the ITR-flanked transgene and the rep/cap genes. The second is based on stable producer cell lines in immortal cancer (HeLa) cells carrying the ITR-flanked transgene and the rep/cap genes. Rep proteins are not expressed, as the HeLa carries no adenoviral genes. However, infection with wild type AAV is required for AAV production. The inclusion of replication-competent viral agents into a production process is a concern that need to be taken seriously.

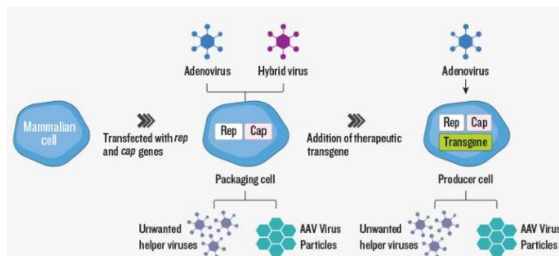

Fig. 6 Production of AAV in mammalian cell lines

Another cell system uses Spodoptera frugiperda cells (Sf9) in combination with baculovirus infection to produce rAAV. The rep/cap genes are stably integrated into the Sf9 cell line genome but are under control of a promoter/enhancer that is induced by subsequent baculovirus infection. In this system, infection can occur with only one baculovirus containing the ITR-flanked gene of interest. Because of their ease of manipulation and their ability to grow to very high cell densities, the Sf9 system has rapidly become a platform of choice for AAV manufacturing. The baculovirus cannot infect mammalian cells, which makes it safer than other viral-based production systems.

The products of AAV production will contain cellular debris (protein/lipids/nucleic acid) and two main types of AAV particles. One with (full capsids) and one without (empty capsids) the transgene. The empty capsids are a contaminant that must be removed or controlled. In small scales, this can be done through ultracentrifugation. This process is non-scalable and cumbersome. For bigger quantities, the AAV's affinity for carbohydrates (affinity chromatography) can be used as an initial capture step in AAV purification to get rid of debris and IEX (ion exchange chromatography) to get rid of empty capsids.

Fig. 7 Centrifuge used for the purification of AAV at the Biozentrum Basel

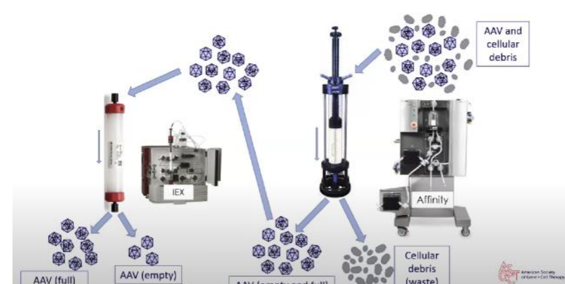

Fig. 8 Usage of affinity and IEX centrifugation for AAV purification

Interview

Summarized Interview

We interviewed Dr. Judith Reinhardt who is a researcher at the "Biozentrum" in Basel for Professor Markus Rüegg and Professor Peter Yurchenco's research team. They do research on AAV based gene therapy. We interviewed her to help us better understand the way these vectors work, how they deliver the DNA into the body, what her and the research team she works with are developing to treat LAMA2 muscle dystrophy, and what the future holds for her field. The main findings from the interview are summarised below.

The AAV vector has a capacity of 4.8 kilobases, while the gene it's carrying is 10 kilobases. To go around this problem, micro genes are created. Viruses are single-stranded, while DNA in eukaryotes is double-stranded, so when AAV vectors are used, they can only deliver single strands. The human cells synthesize the missing strand, but this takes too long. To solve this problem, scientists have invented "self-complimentary" DNA. This is a single strand, which can be carried by the AAV vector, and is twice the size of the single strand. Inside the body, the long strand folds into a double-stranded segment of DNA. Due to this long single strand now needing twice the amount of space in the vector, only 2.4 kilobases of double-stranded DNA are produced in the end, which is not enough to make a difference.

The AAV vector is topped with proteins that are used to dock on the gene, which means that the vector is connected to the gene, so the therapy can be implemented. Due to the vector being viral, the immune system detects it, and the patient becomes immune to it after the first gene therapy treatment using it. Some people are also naturally immune to the AAV vector, which means that prospective patients must be tested for it. The CRISPR/Cas9 system could avoid the immune system problem, but it comes with other dangers. The CRISPR/Cas9 system uses a "gene scissor" that cuts mutations out. In theory this works, but the "scissor" is not precise, which means that the "scissor" could cut out parts of the gene that isn't supposed to be cut out. This could have dire consequences for the patient's body.

10^14 particles per kilogram of the AAV vector enter the body. In muscle dystrophy diseases, the affected areas of the body are everywhere, so locally injecting the therapy would take too long and not be able to be injected everywhere, due to some places being unreachable with a needle. Intravenous therapy solves this problem, because the vectors end up in the bloodstream and are dispersed everywhere throughout the body; even in part it should not be (the liver and other organs). The DNA strands are in these organs, but they don't do anything. Why is this? The AAV vector carries a promoter with it (the SPC512) that only activates the DNA in the places they are supposed to be active. The terminator (in this case the BGH poly A) requires poly A tails to successfully stabilize the RNA from the DNA. Muscle cells don't multiply like cells in other parts of the body, so once the DNA is there, it stays forever. In places where cells multiply regularly, the DNA is diluted over time.

For small animals to test the vectors, small amounts of the AAV vectors can be produced in the lab. To produce them, a centrifuge is used to rid the product of any unwanted, empty particles. For people, labs buy the production of companies that specialize in making amounts for humans, which would need a centrifuge the size of a house.

Companies try to perform as little tests on animals as possible. In Switzerland there are laws against performing tests on primates, while in China the laws aren't as strict. The reason scientists perform tests on animals is that it is difficult to produce a comparable cell culture to one found in animals or humans. Cell cultures that are produced in labs lack things like an immune system. Gene therapies come with a significant price tag. Due to the therapy's profitability, more and more companies are expected to create their own gene therapies, creating competition in the field to drive the prices down. Today, companies like Novartis and SMA have superior gene therapy. They justify their prices with the fact that their therapies only must be done once and last a lifetime, while other companies have created more inexpensive therapies that aren't as efficient and have to be repeated.

Discussion

AAV was discovered 50 years ago and has since become one of the leading gene delivery vectors in clinical development. As a result of its unique biology, simple structure, and no known disease associations, AAV could become the vector of choice for most gene therapy applications. Gene therapy using rAAV has been demonstrated to be safe and well-tolerated in virtually every clinical setting in which it has been used. Among the critical parameters to be considered are vector design, capsid selection, desired target cell and tissue type, and the

route of administration. For the transgene to be delivered optimized for expression, the right AAV variant with an appropriate capsid for target cell transduction and the immunoreactivity profile, and the appropriate delivery approach to maximize target tissue exposure while limiting off-tissue exposure are key focal points for AAV-based therapies. While significant advancements are made, there are still challenges considering effective delivery of rAAV to target tissues and the immunogenicity of rAAV, affecting safety, durability, and efficacy of the gene therapy.

Though it isn't working yet, integrating rAAV with CRISPR-based tools, as gene editing technologies advance, could be a promising strategy for targeted genetic modification.

Up-to-date gene therapy has always been expensive and risky. However, since the FDA has been approving more gene therapies and competition is forming on the market, there is increased interest and possibilities. The FDA has stated that they will continue to fast-track approvals to not halt the distribution of life-saving treatments. Such approach has risks, as specialists fear customers will pay a lot of money for weak and ineffective or even dangerous drugs, but clearly boosts research efforts in this field.

Important questions remain; will the transgene have the desired effect? Is the target cell driving the disease state? Is the turnover rate of the cell high, requiring repeated dosing.

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Picture References:

Fig 1 & 2: Adeno-Associated Virus (AAV) – Production and Modification of AAV | abm Inc. (abmgood.com)
Fig 3: Adeno-Associated Virus (AAV) as a Vector for Gene Therapy by F. Naso et al., BioDrugs (2017) 31:317-334
Fig 4: Dyno Therapeutics Announces Research Published in Science Enabling Artificial Intelligence Approach to Create New AAV Capsids for Gene Therapies - Dyno Therapeutics (dynotx.com)
Fig 5: Sent by Dr. Judith Reinhard from the Biozentrum
Fig 6: A helping hand for gene therapy production (nature.com)
Fig 7: Sent by Dr. Judith Reinhard from the Biozentrum
Fig 8: Lunch & Learn: How AAV Vectors Are Made

1. What is the packaging size of the rAAV vector they are using? Is it necessary to increase this size and if so, do they use dual, overlapping vector strategies or other strategies?

   The vector is 4.8 kb, and the gene is 10 kb, so it is not possible to fit the entire gene in one vector. The gene and the protein must be fitted into micro genes, because you can't make the vectors bigger. This AAV virus isn't naturally present in the body, so after one dose, the person becomes immune. Some people naturally have antibodies, so patients/ subjects must be tested before being injected with them. AAV is topped with proteins that are used to dock (lands on the gene and connects to it) to the gene, which the immune system also detects. People are trying to prevent the immune system from detecting it, but this has proven to be difficult. CRISPR-CAS9 hasn't worked in a person yet because it cuts mutations (gene edits) but the "gene scissor" isn't precise, so if it is used in a person, it is possible that it cuts somewhere where it isn't supposed to, which is dangerous.

2. We have read that the single-stranded AAV delivered transgene must be converted into a double stranded transgene and that this is considered a limiting step in the onset of transgene expression. Why is this considered a limiting step and is it a problem you deal with in your research?

   Normally DNA in eukaryotes is double-stranded. In viruses they are single stranded, so if virus vehicles are used to deliver something in a person, it can only deliver one strand. The human cell can synthesize the other missing strands, which takes time. So, people tried to make it "self-complimentary": the single strand is twice the length of the strand needed and "folds into two strands". Twice the space is needed, which means that half of the capacity is gone. Only about 2.4 KB are possible to bring in, which is too little for humans.

3. What do they use as a promoter and how does this promoter function better than others for their therapy? Is this promoter limited to a certain region because it is silent in certain cell types?

   One challenge is the size of the promoters. The promoter must decide in what cell the virus is active, so a lot of AAV vectors go into different organs. When using an IV, the virus goes to the liver and other organs, which brings in the DNA strand. The promoter decides if it should activate or not. The promoter that is only active in muscles doesn't activate in the liver (for example). The DNA is still in the liver, but the promoter isn't active, so nothing is done, which would cause side effects. In muscle dystrophy sicknesses, all muscles are affected, so if one would try to inject locally, it would take too long, because you would need to inject the vector in every muscle, and some are hard to reach. By using the IV drip, it's distributed everywhere in the body and the promoter decides where it's active.

4. What do they use as a terminator and how does this terminator function better than others for their therapy?

The terminator needs poly A tails to make the rNA from the DNA stabile. We already know it works on humans, so we implement a promoter and terminator and use something that is known to work on humans to test them. Different terminators have been compared and advanced to choose the optimal promoter, so always the “best promoter at this moment in time” is chosen. Today it is the SPC512 (synthetic promoter: designed to be very small, and we use the BGH poly A Terminator, which is a standard in the field.

5. Which cell/tissue type do they target with their therapy?

   We’re trying to get the viruses to only go into the wanted parts of the body (most of them go in the liver). Because of this, there have been people with liver problems. We’re trying to engineer the virus to only go into the liver. The challenge is that the qualities of the AAV vary from species to species. It’s difficult to create something that works 100% of the time on humans. Companies try to do as little studies on primates and other animals as possible. It’s difficult to create the same environment in a cell culture as in an animal (immune system is missing). You can’t reverse the therapy, because the AAV vectors are viruses that live in the person. The virus is not built in (stays out of the chromosome), so it is not built into parts where it shouldn’t be and is diluted overtime. There are cells that don’t multiply a lot or at all (nerve cells and muscle cells), so the DNA in there stays in there forever. Because the muscle cells don’t multiply a lot, one dose could suffice for life and when a muscle grows, the cells that are already there get bigger, so the drug stays.

6. We have read that pre-existing immunity to AAV is thought to be one of the primary factors in early clinical studies failure. Also, we have seen that the capsid proteins and the nucleic acid sequence delivered can trigger components of the immune system and that even if the transgene is a engineered variant the immune system can be triggered. What are you thinking of doing to prevent this from being a factor for your therapy. Are you choosing the serotype of the virus specifically for a patient or is it may be possible to engineer a vector and transgene which doesn't trigger a response?

   The amount of the virus needed is important. You must put in enough viruses that every muscle cell has one particle of the virus, and the amount of it that ends up in the liver must be accounted for. 10^14 particles per kilo enter the body, which also triggers the immune system. The genes that are produced shouldn’t have side effects and be tested to not be immunogenic, so it’s tolerated by the immune system.

7. How do they think to limit off-target tissue exposure to the AAV vectors.

   The promoter is only active in the targeted cells. Certain AAVs don’t go to certain places of the body, so you know there won’t be side effects where it doesn’t go.

8. Do they use systematic or local delivery and how often do they think a patient would have to retake a dose of AAV?

The therapy should be active in every muscle, so it's called systemic (travels through the bloodstream and goes everywhere). By using an IV drip system, it goes into all the muscles, because blood flows there. There are different therapies, where it should only go in the nerve cells, so it's locally injected there, but this doesn't work for the muscles, because (as mentioned before) it must be spread throughout the entire body.

9. How do you purify the AAV. Do you use ultracentrifugation, do you use the affinity of the AAV-specific binding proteins to filter out the empty capsids or do you maybe do something totally different?

   When using a platform, the amount of the virus needed to be produced is considered. Tiny amounts for mice can be produced in the lab, but for people giant tanks are needed, so the production for people is done by companies who are specialized in creating the virus and the production by these companies is bought by researchers and pharmaceutical companies. The viruses are made differently for humans: they must be spotless, so there are no unwanted effects. In the lab it is done with centrifuges, which is the best-established method. Clean AAV particles are yielded, and other unwanted, empty particles are gotten rid of. Companies don't use centrifuges because they would have to be the size of a house.

10. How does restoring the reading frame lead to the production of functional dystrophin?

    The problem with the reading frame is that there are three nucleotides in DNA codes that make up an amino acid. When a patient has a mutation (deletion) where three nucleotides are missing, the reading frame is the same. When one nucleotide is gone, the sequence is nonsense, so people are trying to skip certain bases to get the amino acids back in the order they're supposed to be in. Almost every patient has a different mutation, so when therapies are made for specific sequences, they only work for a handful of patients. You would have to create a separate therapy for almost every person.

11. We have heard that gene therapies are very expensive, do you think they will become affordable soon?

    Gene therapies by Novartis and SMA are very expensive and a one-time thing. The argument of the companies is that production is very expensive and that patients could get different treatments that cost less, but they would have to be repeated. Due to the therapy's profitability, more companies have become interested in these therapies. People believe that over time it should become less expensive because other companies will create their own therapies and a competitive market will result in the prices dropping.