SELINUS UNIVERSITY
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HUMAN GENE THERAPY
(RECOMBINANT AAV SEROTYPE 2)

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Signed electronically by Mr. Somesh R. Doddi
Authenticity statement: I do hereby attest that I am the sole author of this project/thesis and that its contents are only the result of the readings and research I have done.

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Abstract

Adeno-associated virus in it's recombinant form (recombinant adeno-associated virus serotype 2) is one of most promising vectors for human gene therapy.

In this dissertation, I have proposed ten blockbuster disease applications using recombinant adeno-associated virus serotype 2.

ABBREVIATIONS

rAAV2	Recombinant adeno-associated virus serotype 2

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CHAPTER 1

Introduction

Gene therapy is an experimental treatment that involves introducing genetic material into a person's cells to fight or prevent disease. It can be performed on somatic (non-reproductive) cells and is not passed on to future generations, but is short-lived. It can also be performed on germline cells (gene engineering passed on to future generations) in humans which is controversial and prohibited due to ethical reasons. A gene can be delivered to a cell using a carrier known as a “vector.”

Target cells are infected with the vector. The vector then unloads its genetic material containing the therapeutic human gene into the target cell. The generation of a functional protein product from the therapeutic gene restores the target cell to a normal state.

Somatic gene therapy can be broadly split into two categories: “ex vivo”, which means exterior (where cells are modified outside the body and then transplanted back in again). In some gene therapy clinical trials, cells from the patient's blood or bone marrow are removed and grown in the laboratory. The cells are exposed to the virus that is carrying the desired gene. The virus enters the cells and inserts the desired gene into the cells' DNA. The cells grow in the laboratory and are then returned to the patient by injection into a vein. This type of gene therapy is called ex vivo because the cells are treated outside the body “in vivo”, which means interior (where genes are changed in cells still in the body). This form of gene therapy is called in vivo, because the gene is transferred to cells inside the patient's body.

Here, we are employing “in vivo” gene therapy.

CHAPTER 2

I. The current set of technologies on offer

Vector systems can be divided into: Viral Vectors - These include retroviruses, adenoviruses, adeno-associated viruses, lentiviruses, pox viruses, alphaviruses, and herpes viruses. These viruses differ in how well they transfer genes to the cells they recognize and are able to infect, and whether they alter the cell's DNA permanently or temporarily.

Here, we are employing "in vivo" gene therapy.

		Adenovirus	Adeno-associated virus	Alphavirus	Herpesvirus	Retrovirus / Lentivirus	Vaccinia virus
Particle characteristics	Genome	dsDNA	ssDNA	ssRNA (+)	dsDNA	ssRNA (+)	dsDNA
	Capsid	Icosahedral	Icosahedral	Icosahedral	Icosahedral	Icosahedral	Complex
	Coat	Naked	Naked	Enveloped	Enveloped	Enveloped	Enveloped
	Virion polymerase	Negative	Negative	Negative	Negative	Positive	Positive
	Virion diameter	70 - 90 nm	18 - 26 nm	60 - 70 nm	150 - 200nm	80 - 130 nm	170 - 200 X 300 - 450nm
	Genome size	39 - 38 kb	5 kb	12 kb	120 - 200 kb	3 - 9 kb	130 - 280 kb
							
	Family	*Adenoviridae*	*Parvoviridae*	*Togaviridae*	*Herpesviridae*	*Retroviridae*	*Poxviridae*
Gene Therapy Properties	Infection / tropism	Dividing and non-diving cells	Dividing and non-diving cells	Dividing and non-diving cells	Dividing and non-diving cells	Dividing cells*	Dividing and non-diving cells
	Host genome interaction	Non-integrating	Non-integrating*	Non-integrating	Non-integrating	Integrating	Non-integrating
	Transgene expression	Transient	Potential long lasting	Transient	Potential long lasting	Long lasting	Transient
	Packaging capacity	7.5 kb	4.5 kb	7.5 kb	> 30 kb	8 kb	25 kb

A comparison of different viral vectors in use for gene therapy: overview of their advantages and disadvantages. *NOTE: Adeno-associated viruses are able to integrate with low frequency into chromosome 19 (68%). Lentiviruses also infect

non-dividing cells.

From the table and the NOTE we can conclude that the adeno-associated virus (aav) is the only virus which can affect dividing and non-dividing cells, integrate in a site-specific manner into human chromosome 19 in a long lasting manner (estimated efficacy 10-12 years per dose administered). Immunologically, aav causes a very mild immune response in the body. Lentiviruses behave very similar to aav, but the problem with lentiviruses is that they integrate randomly into the human genome (chromosome), causing undesirable side effects such as mutations, and/or cancer.

Non-viral Vectors- Non-viral methods present certain advantages over viral methods, with simple large scale production and low host immunogenicity being just two. Previously, low levels of transfection and expression of the gene held non-viral methods at a disadvantage; however, recent advances in vector technology have yielded molecules and techniques with transfection efficiencies similar to those of viruses.

Naked DNA- This is the simplest method of non-viral transfection. Clinical trials carried out of intramuscular injection of a naked DNA plasmid have occurred with some success; however, the expression has been very low in comparison to other methods of transfection. In addition to trials with plasmids, there have been trials with naked PCR product, which have had similar or greater success. This success, however, does not compare to that of the other methods, leading to research into more efficient methods for delivery of the naked DNA such as electroporation and the use of a "gene gun", which shoots DNA coated gold particles into the cell using high pressure gas.

Oligonucleotides- The use of synthetic oligonucleotides in gene therapy is to

inactivate the genes involved in the disease process. There are several methods by which this is achieved. One strategy uses antisense specific to the target gene to disrupt the transcription of the faulty gene. Another uses small molecules of RNA called siRNA to signal the cell to cleave specific unique sequences in the mRNA transcript of the faulty gene, disrupting translation of the faulty mRNA, and therefore expression of the gene. A further strategy uses double stranded oligodeoxynucleotides as a decoy for the transcription factors that are required to activate the transcription of the target gene. The transcription factors bind to the decoys instead of the promoter of the faulty gene, which reduces the transcription of the target gene, lowering expression..

Lipoplexes and polyplexes- To improve the delivery of the new DNA into the cell, the DNA must be protected from damage and its entry into the cell must be facilitated. To this end new molecules, lipoplexes and polyplexes, have been created that have the ability to protect the DNA from undesirable degradation during the transfection process.

Plasmid DNA can be covered with lipids in an organized structure like a micelle or a liposome. When the organized structure is complexed with DNA it is called a lipoplex. There are three types of lipids, anionic (negatively charged), neutral, or cationic (positively charged). Initially, anionic and neutral lipids were used for the construction of lipoplexes for synthetic vectors. However, in spite of the facts that there is little toxicity associated with them, that they are compatible with body fluids and that there was a possibility of adapting them to be tissue specific; they are complicated and time consuming to produce so attention was turned to the cationic versions. Cationic lipids, due to their positive charge, naturally complex with the negatively charged DNA. Also as a result of their charge they interact with the cell

membrane, endocytosis of the lipoplex occurs and the DNA is released into the cytoplasm. The cationic lipids also protect against degradation of the DNA by the cell. The most common use of lipoplexes has been in gene transfer into cancer cells, where the supplied genes have activated tumor suppressor control genes in the cell and decrease the activity of oncogenes. Recent studies have shown lipoplexes to be useful in transfecting respiratory epithelial cells, so they may be used for treatment of genetic respiratory diseases such as cystic fibrosis.

Complexes of polymers with DNA are called polyplexes. Most polyplexes consist of cationic polymers and their production is regulated by ionic interactions. One large difference between the methods of action of polyplexes and lipoplexes is that polyplexes cannot release their DNA load into the cytoplasm, so to this end, co-transfection with endosome-lytic agents (to lyse the endosome that is made during endocytosis, the process by which the polyplex enters the cell) such as inactivated adenovirus must occur. However this isn't always the case, polymers such as polyethylenimine have their own method of endosome disruption as does chitosan and trimethylchitosan.

For gene therapy, animal testing (rat, mouse, monkey) is first performed before any clinical trials are conducted in humans.

II. The problem with the current set of technologies on offer

Viral vectors beat non-viral vectors on two points; SPECIFICITY and GENE EXPRESSION LEVELS. Aav serotype 2 (aav2) is the most popular and commonly studied (and published) aav, and we would like to develop recombinant adeno associated virus serotype 2 (raav2) vectors for application in human gene therapy. The genomic structure consists of a replication (rep, left) and capsid (cap, right) gene flanked by two inverted terminal repeats (ITR's). The rep gene is important for integration of wild type aav onto a specific site on human chromosome 19. Important point: Advisable not to delete the rep gene because it plays a role in latency and site specific chromosomal integration. It is alright to delete the cap gene and replace it with the therapeutic transgene.

Our goal is to create recombinant aav (raav serotype 2 which is the most common, raav2) in which the cap gene (and situationally the rep gene) are deleted and replaced by the gene of interest (transgene).

Aav has some drawbacks, but these drawbacks can be remedied. Limited transgene carrying capacity- Aav2 has a limited transgene carrying capacity; accomodated transgene less than or equal to 4.5 kb.

Solution: Look for situations where the transgene insert is of a small size so that there is minimal disruption in the layout of rep gene in the aav2 genome. Within the restrictive transgene capacity of maximum 4.5 kb, I have ten proposed blockbuster experiments/applications of recombinant aav2 (raav2) at http://doddisnobelnomination1.blogspot.in/ for human gene therapy and it is likely that any of them could be a breakthrough because the science I have been discussing here is in agreement. In my proposed experiments, I tackle the following conditions:

Human lung cancer and Human liver cancer (quiescence)

For lung cancer: The gene delivery system to be used will be adeno-associated virus helper-free system: raav2/5 or maybe 2/6 hybrid. This recombinant virus has genetic elements of the aav2 genome (chromosome 19 site specific integration) and aav5 capsid (lung specificity). For liver cancer: The gene delivery system to be used will be adeno-associated virus helper-free system: raav2/3 or maybe 2/8 hybrid. This recombinant virus has genetic elements of the aav2 genome (chromosome 19 site specific integration) and aav3 capsid (liver specificity). Interest in isolating and determining the "cell re-programming (cancerous cells) genetic sequence" with the preferable characteristic of it being 'a ss 1.5-maximum 3kb dna insert'. This sequence will serve as the gene of insert (goi) for the study.

After the human development phase, most cells in the human body reach a state of 'quiescence' (G0 phase, referred to the G zero phase or resting phase) and do not divide further, by exiting the cell cycle. Quiescent cells can re-enter the cell cycle. The development of tumorigenic (cancerous) cells occurs when they bypass quiescence and proliferate. In the typical cell cycle of G1-R-late G1-S-G2-M; there is indication that loss of regulation in 'R' checkpoint would allow cells to enter the cycle in the absence of mitogenic signalling, leading to unscheduled proliferation. While it may be unfeasible to completely convert cancerous cells to normal cells, we would want to 'arrest' the proliferation of cancerous cells by introducing a gene of interest (goi) into them that will convert them into quiescent cells. In the G0 phase, it has been found that a protein which is a member of the retinoblastoma family viz. P130 binds to the DP-E2F protein transcription factor and inactivates it. The p130/RBL2 (retinoblastoma-like protein 2) is a 130 kDa protein. Using the protein/DNA conversion table, this equates to 3.51 kb dna, which is slightly above

our 3kb dna insert limit into adeno-associated virus helper free gene delivery system. However, it is indicated that the p130 gene has been successfully integrated into aav, and it is non pathogenic, non toxic and not immunogenic and raav can accomodate inserts from 3-4.9 kb. The p130 gene is a potent inhibitor of E2F mediated transcription activation, and may act as a tumor suppressor. In a study, the gene was inserted into retrovirus vehicle and introduced into the human lung cancer cell line in mice. It was found to suppress tumor growth. The p130 gene has been obviously isolated and it's genomic structure has been determined. P130 is a “bona fide” tumor suppressor gene and it may be a candidate for cancer gene therapy for lung cancer (in combination with adeno-associated virus helper-free system vector: raav2/5 hybrid) and liver cancer (in combination with adeno-associated virus helper-free system vector: raav2/3 hybrid).

Conclusion: The p130 tumor suppressor gene will be the gene of interest (goi) in our study, and we would like to insert it in the gene delivery system/vector of helper-free adeno-associated virus. Thereon, we will inject (into bloodstream: intravenous or intraarterial; or intramuscular) this recombinant adeno-associated virus into the central nervous system of a cancer cell line patient (lung cancer and liver cancer) and hope for integration of the virus with the gene of interest onto the specific site on chromosome 19. The p130 gene will be expressed from chromosome 19, and the p130 protein will bind to and inactivate the DP-E2F protein transcription factor and basically arrest the proliferation of the cancerous cells by converting them into quiescent cells.

Human lung cancer and Human liver cancer (apoptosis)

High-energy rays (radiation) damage DNA in cells, causing them to die or stop dividing. Since cancer cells divide more frequently than normal cells, they are more

susceptible to damage. Healthy cells can be affected as well but are better able to repair the damage. [3H]-thymidine labelling of DNA triggers apoptosis potentiated by E1A-adenoviral protein. E1A-adenoviral protein is a 32 kDa protein, which equates to 0.864 kb DNA (gene of interest) per standard protein/dna conversion table. I propose [3H]thymidine labelling of the viral genome of helper-free recombinant adeno-associated virus raav 2/5 or maybe 2/6 hybrid (for lung cancer) and raav 2/3 or maybe 2/8 hybrid (for liver cancer).

This will make all the thymidine nucleotides in the viral genome radioactive. Recall; recombinant raav 2/5 hybrid- This recombinant virus for lung cancer treatment has genetic elements of the aav2 genome (chromosome 19 site specific integration) and aav5 capsid (lung specificity). Recombinant raav 2/3 hybrid- This recombinant virus for liver cancer treatment has genetic elements of the aav2 genome (chromosome 19 site specific integration) and aav3 capsid (liver specificity). Then, the gene of interest viz; 0.864 kb dna of E1A-adenovirus is inserted in radioactive recombinant adeno-associated virus raav 2/5 hybrid (for lung cancer) and raav 2/3 hybrid (for liver cancer). After injection (into bloodstream: intravenous or intraarterial; or intramuscular) of virus into cancer patient, the virus will hopefully integrate onto human chromosome 19 at a specific site, causing portion of chromsomal dna to be radioactively labelled. Upon expression of E1A-adeno viral gene of interest, apoptosis will be triggered in presence of radioactive thymidine. As mentioned above, apoptosis will be higher in cancer cells (lung and liver); while healthy cells will be better able to repair dna damage caused by radiation (radioactive thymidine). Thus, the cancer cells in the lung and liver will be selectively destroyed using small doses of radiation at the nuclear dna (chromsosomal) level.

Conclusion: We have developed a virus that can induce apoptosis at the nuclear dna(chromosomal) level by selectively killing cancer cells (lung cancer and liver cancer) using a combination of radioactive thymidine and expressing E1A-adenoviral protein, and this is useful for targeted cancer treatment.

Human AIDS (HIV)

HIV/AIDS is a retroviral disease with a high fatality rate in humans. After reading a plethora of scientific publications and seeing the progress made to date, I have concluded to identify and devise an experimental design for it's treatment. The HIV-1 integrase protein plays a crucial role in generating hydroxyl ends on the 3' end of the viral DNA (after action of reverse transcriptase) within the cytoplasm. Integrase also forms a complex with the viral DNA and after transport into the cell nucleus, HIV-1 integrase acts as a catalyst in ligating viral DNA into host DNA at a random site on a random chromosome by the hydroxyl group on the viral DNA attacking the 5' phosphate end on the host DNA (chromosome) by formation of a phosphodiester bond. Now, the viral DNA is fully integrated into the host DNA. An important area of research is the discovery and development of integrase inhibitors. Diketo acids are potent inhibitors of HIV-1 integrase that inhibit integration and viral replication in cells. A protein from Cornebacterium species which is a diketo acid is 2,5-diketo-D-gluconic acid reductase A, 277 amino acids long which equates to 0.831 kB dna or a 30.77 KDa protein (this is our gene of interest). Interestingly, this protein also catalyzes the conversion of 2,5-diketo gluconic acid (obtained by conversion of d-glucose using a prokaryotic microorganism) to 2-keto l-gluconic acid which is then converted to l-ascorbic acid (vitamin c) in the human body. Next, I propose using the gene delivery of adeno-associated virus helper-free system:

recombinant raav2. This recombinant virus has genetic elements of the aav2 genome (chromosome 19 site specific integration), and will accomodate the 0.831 KB dna of 2,5-diketo-D-gluconic acid reductase A (gene of interest). Other promising methods for treatment of HIV/AIDS include administering of radiation, induction of apoptosis by use of "executioner protein" caspase-6, and possibly some method of insertion of PEST protein sequence as a signal peptide for protein degradation onto the translated viral polypeptide after it exits the cell nucleus and exists in the cytoplasm.

Conclusion: 2,5-diketo-D-gluconic acid reductase A will be the gene of interest (goi) in our study, and we would like to insert it in the gene delivery system/vector of recombinant helper-free adeno-associated virus 2 viz; raav 2. Thereon, we will inject (into bloodstream: intravenous or intraarterial; or intramuscular) this recombinant adeno-associated virus into the central nervous system of a HIV/AIDS patient and hope for integration of the virus with the gene of interest onto the specific site on chromosome 19. The 2,5-diketo-D-gluconic acid reductase A gene will be expressed from chromosome 19, there will be an increase in the cellular level of 2,5-diketo-D-gluconic acid reductase A protein, and we would expect an inhibition in HIV-1 integrase. This will prevent the chelation and formation of hydroxyl groups on the 3' ends of the viral DNA, and also prevent ligation and integration of viral dna into the host genome (ie host chromosome), thereby affecting the infectivity of the virus. These steps will be beneficial in the treatment of HIV/AIDS at the nuclear dna ie chromosomal level.

Human Insulin resistant diabetes (Type 2 diabetes)

Insulin resistance (IR) is a physiological condition where the natural hormone

insulin becomes less effective at lowering blood sugars. The resulting increase in blood glucose may raise levels outside the normal range and cause adverse health effects, depending on dietary conditions. Certain cell types such as fat and muscle cells require insulin to absorb glucose. When these cells fail to respond adequately to circulating insulin, blood glucose levels rise.

The liver helps regulate glucose levels by reducing its secretion of glucose in the presence of insulin. This normal reduction in the liver's glucose production may not occur in people with insulin resistance. In mice, it is found that endogeneous glucose production is inhibited by purified, recombinant Acrp30 protein. The mode of delivery is intraperitoneal injection. The study concludes that an acute increase in circulating Acrp30 levels lowers hepatic glucose production without affecting peripheral glucose uptake. Thus, a moderate rise in circulating levels of the adipose-derived protein Acrp30 inhibits both the expression of hepatic gluconeogenic enzymes and the rate of endogenous glucose production. In humans, the Acrp30 gene is located on chr. 3q27. It is a 30kDa protein, whcih equates to 0.81 kb dna per standard dna/protein conversion table. Thus, we can conclude that increasing the cellular concentration of Acrp30 protein shows promise for lowering endogenous glucose production, without lowering peripheral glucose uptake, and this results in decreased blood glucose concentration; preventing the onset of type 2 diabetes. So, increased cellular Acrp30 protein levels can be beneficial as a treatment for insulin resistant diabetes in muscle, fat, and liver cells. Accordingly, I postulate the use of a recombinant adeno-associated virus 2 (serotype 2) raav2 vector, in which the 0.81 kb dna fragment for human Acrp30 gene (gene of interest) will be inserted.

Conclusion: The Acrp30 gene will be the gene of interest (goi) in our study, and we would like to insert it in the gene delivery system/vector of recombinant helper-

free adeno-associated virus 2 viz; raav 2. Thereon, we will inject (into bloodstream: intravenous or intraarterial; or intramuscular) this recombinant adeno-associated virus into the central nervous system of an insulin resistant diabetic patient and hope for integration of the virus with the gene of interest onto the specific site on chromosome 19. The Acrp30 gene will be expressed from chromosome 19, there will be an increase in the cellular level of Acrp30 protein, and we would expect a decrease in endogenous glucose production, without lowering peripheral glucose uptake, and thereby resulting in decreased blood glucose concentration. These steps will be beneficial in the treatment for insulin resistant diabetes in muscle, fat, and liver cells; and will eliminate type 2 diabetes at the nuclear dna ie chromosomal level.

Human aging (extending the Hayflick limit)

I have come up with a serious design for human immortality that will utilize adeno-associated virus (serotype aav 2) as a vector and that will deliver therapeutic gene of interest viz; catalytic subunit of telomerase (hTERT) which is 1132 aa long/126kDa/3.43 kB to normal cells of the central nervous system via intravenous or intraarterial delivery or intramuscular injection. The gene of interest will bind to and form a concatemer at a specific site on human chromsome 19. For forming the recombinant adeno-associated virus vector (serotype aav2) the following is required for the transfection process: Human Embryonic Kidney Cells, HEK-293 Plasmid of aav2 with hTERT therapeutic gene inserted Plasmid pRep2Cap9- This is a helper plasmid with aav serotype 2 rep gene, and aav9 cap gene (CNS specificity) Adenovirus helper factors When the catalytic component (i.e. hTERT) of the telomerase holoenzyme is transfected into cells, its expression leads to maintenance of the telomere end-regions of chromosomes. In most instances, this suppresses

replicative senescence and causes the cells to become immortal.

Human cystic fibrosis, 'biofilm'

Recombinant adeno-associated virus serotype 5 (raav-5) has been shown to be useful in treating cystic fibrosis. However, in cystic fibrosis patients there is a 'biofilm' of thick mucus blocking the airway consisting of proteins called defensins secreted by bacteria such as Pseudomonas aeruginosa.

As a result, the recombinant adeno-associated virus serotype 5 (raav-5) (see http://www.doddisnobelnomination.blogspot.com for background on aav; raav-2) containing the shortened cftr cDNA gene cannot get through the airway epithelial cells to the nucleus of the cell into the chromosome to integrate and replace the faulty cf gene. Delivery is the issue in cf patients due to blockage by biofilm.

During the construction of the raav-5 construct, I propose inserting the 0.405kb (15 kDA protein) staphylokinase gene (from Staphylococcus aureus) as it has the ability to neutralize defensins, and clear the biofilm. Now, raav-5 can enter the body and act.

Human induced pluripotent stem cells- new generation (IPSC's was a nobel prize winner!) Reprogramming of human cells to iPSCs was reported in November 2007 by two independent research groups: Shinya Yamanaka of Kyoto University, Japan, who pioneered the original iPSC method, and James Thomson of University of Wisconsin-Madison who was the first to derive human embryonic stem cells.

With the same principle used in mouse reprogramming, Yamanaka's group successfully transformed human fibroblasts into iPSCs with the same four pivotal genes, OCT3/4, SOX2, KLF4, and C-MYC, using a retroviral system, while Thomson and colleagues used a different set of factors, OCT4, SOX2, NANOG, and LIN28, using a lentiviral system.

Yamanaka created the 'gold standard' and won the Nobel Prize (alongwith Dr. John Gurdon).

Building on Yamanaka's work, the OCT3/4, SOX2, KLF4, and C-MYC gene have the following gene sizes:

OCT3/4: 1.042 kb (38.571 kDA)

SOX2: 2.513 kb (92.981 kDA)

KLF4: 5.631 kb (208.35 kDA); human cdna 1.459 kb (54 kDA)

C-MYC: 1.319 kb (48.804 kDA)

The problem was the iPSCs had the retroviral or lentiviral gene integrate randomly into the human fibroblast cell chromosome, causing undesirable side effects.

I propose a safe recombinant adeno-associated virus that is applicable (raav-2) for creating human induced pluripotent stem cells from human fibroblasts which can accommodate the oct3/4, sox2, c-myc, and klf4 gene.

Raav-2 integrates into human chromosome-19 at AAVS1 and this site is found to be a 'safe harbor' with no genotoxic effects on the cells. If successful experimentally (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3347549/, see paragraphs on human adipose stem cells, human keratinocytes), this will elevate the generation of induced pluripotent stem cells (iPSCs) to a new level.

Human Type 1 diabetes

The human insulin protein is composed of 51 amino acids, and has a molecular mass of 5808 Da; which equates to 0.157 kB.

I have already proposed a safe recombinant adeno-associated virus that is applicable (raav-2) for creating human induced pluripotent stem cells from human fibroblasts which can accommodate the oct3/4, sox2, c-myc, and klf4 gene.

Raav-2 integrates into human chromosome-19 at AAVS1 and this site is found to be a 'safe harbor' with no genotoxic effects on the cells

(http: //www.sciencedirect.com/science/article/pii/S152500161630377X).

If successful experimentally, this will elevate the generation of induced pluripotent stem cells (iPSCs) to a new level

(https: //doddisnobelnomination4.blogspot.in/;

https: //www.ncbi.nlm.nih.gov/pmc/articles/PMC3318626/).

Next, I propose creation of another recombinant adeno-associated virus ins (raav-2 ins; or another raav serotype based on antibody effect) in which only the 'cap' (capsid) gene is replaced with 0.157 kB of the insulin protein gene. This virus is then to be administered to the induced pluripotent stem cells (iPSCs); so that raav-2 ins can integrate into the iPSCs into human chromosome-19 at AAVS1. These iPSCs can then be injected into the pancreas of the patient (raav-2 can be made antibody evasive, reference available upon request), so that the patient can start producing insulin. Choice of the right human insulin promoter will be important (to be included during construction of raav-2 ins), for precise control of insulin production; such as the glucagon-like peptide-1 (GLP-1, 2.3 kB) promoter (https://bmcbiotechnol.biomedcentral.com/articles/10.1186/1472-6750-11-99).

Human wound healing

Human wound healing is a serious problem. Keratinocyte growth factor (kgf-2; also formerly known as fibroblast growth factor fgf-12) can therapeutically promote or accelerate wound healing.

I propose the insertion of kgf-2 (208 amino acids, 23.09 kDa, 0.624 kB) gene into the capsid (cap) region to create recombinant adeno-associated virus serotype 2

(raav-2) which can be administered to the patient for accelerated wound healing. The gene gets integrated into a particular site on human chromosome 19 (AAVS1), and leads to permanent genetic medicine. It is hoped after administration of raav-2 that the patient's wound healing rate is either restored or permanently improves. Human adaptive immune response strengthening (T-cells) IL-2 is a cytokine that contributes to the differentiation and survival of regulatory T cells, thereby ensuring their significance in the control of the immune response and their effective participation in the pathogenesis of several pathological conditions, such as cancer and metabolic, infectious, autoimmune and inflammatory diseases.

The il-2 gene is 5.256 kB (194.47 kDA protein; cdna of il-2 gene is 0.462 kb), and I propose inserting it into recombinant adeno-associated virus serotype-2 (raav-2). This way, the il-2 gene will get integrated into human chromosome 19 (site aavs1), and lead to permanent genetic medicine as well as greater control of the adaptive immune response through T cells.

Aav susceptible to attack and neutralization by the human body's 'neutralizing antibodies' (NAB)- As a result, the 'cell mediated response' and 'humoral response' are triggered in a complex cascade of events.

Solution: The toll like receptor (tlr) ligands (information is confidential) on recombinant aav2 have to be deleted, and the empty space has to be filled with a 'dna repeating motif'. It is important to note that the deletion of tlr's makes aav2 antibody evasive (scientific citation available).

III. How my invention attempts to solve the problem

In my invention, the following points should be borne in mind:

- Wild type aav2 integrates onto a specific locus on human chromosome 19. So does recombinant aav, raav2.

- A breakthrough: I have identified a chromosome (information is confidential), where raav2 has a high specificity to integrate onto. This was previously confirmed by US patent US5773289 A (not filed by me).

- When the cap (and situationally the rep gene) are removed from wild type aav2, the empty space I have discovered has to be filled with a 'dna repeating motif' (information is confidential) which is complementary to the sequence on the chromosome mentioned above; for integration.

- Note: Experimental binding studies will have to be performed to verify that the raav2 virus is binding to the specific chromosome mentioned above. This opens the door to permanent genetic medicine (estimated efficacy 10-15 years/dose).

- After the tlr's on raav2 have been deleted, the empty space filled in with a 'dna repeating motif', and the cap (and situationally rep gene) gene has been deleted and replaced with the therapeutic transgene, a triple transfection is performed on HEK293 (human embryonic kidney) cells.

- In the triple transfection, the raav2 virus, rep gene (plasmid), cap gene (plasmid), adenovirus helper factors (plasmid) and HEK293 cells is mixed together and incubated.

- The triple transfection gives rise to raav2 viral particles (called virions).

- These raav2 viral particles are purified and then delivered by various routes into the human body for gene therapy.

IV. The technical action methodology in my invention (solution)

It is certain that wild type adeno-associated virus (aav) integrates onto a specific site on chromosome 19 and exhibits latent infection. Recombinant aav (raav) is found to be non-integrating and exist predominantly as circular episomes in the ribosomal dna within the nucleus.

My raav has a very small gene insert size of 0.8kb for the majority of the experiments (with the exception of the p130 gene which is 3.5 kb and a bona fide tumour suppressor), and so during generation of raav there is minimal disruption (deletion) of wild type rep 58, rep 68 and cis-acting elements within the rep gene which are important for chromosomal integration into chromosome 19 and establishing a latent infection.

Hence, my raav's although recombinant behave like wild type aav and integrate into chromosome 19 at a site aav s1 and are able to establish a latent infection.

Hence, my postulated experiments have a high chance of success.

V. How the technical action methodology in my invention (solution) is technically superior to known solutions

Note 1: AAV Integration onto chromosome 19.

Aav chromosomal integration for latency infection requires conservation of the Rep gene elements Rep 68 (or)/78, inverted terminal repeats (cis-acting elements) on aav or recombinant aav (raav), and AavS1 integration site on chromosome 19. This really means that to generate raav the gene insert size is smaller than 3.5 kB, generated by incorrectly removing the rep and cap gene from wild type aav. The gene insert for raav is very small. The AavS1 locus was originally defined in Genomics by Kotin, Menninger, Ward, and Berns. The AavS1 integration site on chromosome 19 is found only in humans and the rhesus macaque monkey. Hence, the rhesus macaque is suitable for pre-clinical studies/research and the human is suitable for clinical studies.

Note 2: Gene inserts into recombinant adeno associated virus (aav).

Gene inserts less than 1 kb: These gene inserts can be accomodated into the cap gene region of wild type aav, generating recombinant aav with the gene of interest. The purified recombinant aav is available for applications.

Gene inserts greater than 1 kb: These gene inserts are accomodated into the rep and cap gene region of wild type aav, generating recombinant aav with the gene of interest. Because the rep gene is required for site specific integration onto chromosome 19, it is to be provided in trans resulting in a mixture of purified recombinant aav + rep gene available for applications.

Note 3: Effect of neutralizing antibodies (B and T cells) on administered recombinant aav

To minimize the effect of neutralizing antibodies, tissue specific promoters on the raav and administering the raav during enzyme replacement therapy is practiced.

Virus infection in vertebrates results in two general types of immune response. The first is a rapid-onset "innate" response against the virus, which involves the synthesis of proteins called interferons and the stimulation of "natural killer" lymphocytes. In some cases, the innate response may be enough to prevent a large scale infection. However, if the infection proceeds beyond the first few rounds of viral replication, the "adaptive immune response", kicks into high gear. The adaptive immune response itself has two components, the humoral response (the synthesis of virus-specific antibodies by B lymphocytes) and the cell-mediated response (the synthesis of specific cytotoxic T lymphocytes that kill infected cells). Both of these components of the adaptive immune response result also in the production of long-lived "memory cells" that allow for a much more rapid response (i.e., immunity) to a subsequent infection with the same virus. The early, nonspecific responses (nonspecific inhibition, natural killer cell activity, and interferon) limit virus multiplication during the acute phase of virus infections. The later specific immune (humoral and cell-mediated) responses function to help eliminate virus at the end of the acute phase, and subsequently to maintain specific resistance to reinfection. Initial antigen presentation by "Antigen Presenting Cells" leads to the activation and proliferation of TH cells (helper T cells), which are required for the generation of the humoral response (clonally-selected B cells secreting antigen-specific antibody that binds to extracellular virus particles) and the cell-mediated response (clonally-selected TC cells recognizing antigen-

displaying "altered self" (i.e., infected) cells and killing them). A subset of these B and TC cell populations become antigen-specific "memory" cells to provide long-lived immunity to re-infection.

Both humoral and cell mediated memory for adeno-associated virus serotype 2 (aav-2) is present in the human population. Pre-existing immunity to wild type aav in humans is pre-dominantly humoral. Genetic modification of aav-2 to escape immune recognition by T cells fully is not a feasible goal.

Transient immunosuppression has promise in mitigating cellular and humoral responses induced by vector application in naïve hosts, but cannot overcome the problem that pre-existing neutralizing antibodies pose towards the goal of safe and efficient gene delivery. Immune suppression is an effective strategy to mitigate the body's immune response long enough to allow the capsid proteins to clear from the cell surface and prevent the formation of neutralizing antibodies in order to facilitate re-administration of the vector. Transient immunosuppression is not a solution to pre-existing neutralizing antibodies to the aav capsid. Modifications, either chemical modifications that protect surface exposed parts of the protein capsid or genetic modifications that result in changes to the protein capsid must be made to the vector to evade these neutralizing antibodies as best as possible. Aav vectors can be altered to decrease antibody neutralization, while still effectively delivering genetic material both in vitro and in vivo. As such, anti-aav antibodies have neutralizing effects that decreases the efficiency of in-vivo gene therapy and can prevent vector readministration. Aav vectors elicit both cellular and humoral response against the transgene product (gene of interest); Aav vectors can escape immunity by capsid modification, use of alternative serotypes, and immunosuppression eg: mycophenolate mofetil, tacrolimus.

In another approach Aav can be made to evade pre-existing neutralizing antibodies (NAB's) in the body by the creation of aav chimeras eg: AAV-DJ. Vector capsid modification is a positive alternative to administration of immunosuppressive drugs. In this area, CpG-depleted aav vectors evade immune detection.

The CpG sites or CG sites are regions of DNA where a cytosine nucleotide is followed by a guanine nucleotide in the linear sequence of bases along its 5' → 3' direction. These findings suggest that AAV-directed immune responses may be circumvented by depleting the ligand for TLR9 (CpG sequences) from the vector genome. Indeed, we found that CpG-depleted AAVrh32.33 vectors could establish persistent transgene expression, evade immunity, and minimize infiltration of effector cells (8). Additionally, there is indication that TLR2 (capsid) has to be deleted also.

Note 4: An important note on recombinant aav (raav) vector re-administration.

For a greater impact in terms of human gene therapy, raav is re-administered. Typically, upon re-administration one would expect a host immune response to the virus. Transient immunosuppression of mice by treatment with antibody to CD4 at the time of primary infection allowed transgene expression after readministration of rAAV vectors to animals. Re-administration of rAAV can be accomplished by down regulating the anti-AAV immune response and suggest the use of repeated administration of rAAV as a viable form of therapy for the treatment of chronic diseases. Successful re-administration of adeno-associated virus vectors to the mouse lung requires transient immunosuppression during the initial exposure by using MR1 and CTLA4Ig and is transient; whether repeat transduction by aav

vectors will be possible in humans is yet unknown.

Note 5: Increasing the frequency of aav/raav onto chromosome 19.

Within the cap gene of raav, our gene of interest in most cases is 0.8 kb. The empty space in the cap gene (flanking the gene of interest) is inserted (occupied) by ribosomal DNA (rDNA) sequences capable of homologous recombination into genomic rDNA.

CHAPTER 3

ADDITIONAL PERSONAL NOTES

I Additional personal notes

Reference: http://www.doddisnobelnomination1.blogspot.com/

Gene of interest is greater than 0.83 kb: This pertains to cell re-programming (quiescence) for lung cancer and liver cancer as the first step of treatment using a 3.51 kb insert (gene of interest). For adeno-associated virus (aav) conversion to recombinant adeno-associated virus (raav) the rep and cap genes are removed from aav2 (serotype 2), CpG (tlr9 ligand) is deleted from aav2 in order to evade immune detection (Additionally, there is indication that TLR2 (capsid) has to be deleted also), 3.51 kb insert (gene of interest) is attached into the deleted repcap region, and the gene of interest is flanked on either side by occupying the free space by ribosomaldna (rdna) homologous sequences as repeating motifs to help in site specific aavs1 integration. Next, the deleted rep and cap genes are provided in trans on a separate plasmid as rep2 cap 5 or 6 (lung cancer) cap 3 or 8 (liver cancer); also the adenovirus helper factor genes are provided in trans on a separate plasmid for the triple transfection of hek293 cells to generate our virus of interest. Optionally, the free space can be occupied by CA (cytosine-adenine) repeating motifs which are complementary to the GT (guanine-thymine) rich binding complementary sequence found on human chromosome 22 for germline integration, hoping for integration of raav into human chromosome 22.*

Gene of interest is less than or equal to 0.83 kb: This pertains to all other

experiments and variations therein using a 0.83 kb insert (gene of interest). For adeno-associated virus (aav) conversion to recombinant adeno-associated virus (raav) the cap gene is removed from aav2 (serotype 2), CpG (tlr9 ligand) is deleted from aav2 in order to evade immune detection (Additionally, there is indication that TLR2 (capsid) has to be deleted also), 0.83 kb insert (gene of interest) is attached into the deleted cap region, and the gene of interest is flanked on either side by occupying the free space by ribosomaldna (rdna) homologous sequences as repeating motifs to help in site specific aavs1 integration. Next, the deleted cap gene is provided in trans on a separate plasmid as cap 'x' ('x' is varying serotype number based on the targeted tropism) only and no repetition of rep gene as excessive expression of rep gene is found to be toxic; also the adenovirus helper factor genes are provided in trans on a separate plasmid for the triple transfection of hek293 cells to generate our virus of interest.

Optionally, the free space can be occupied by CA (cytosine-adenine) repeating motifs which are complementary to the GT (guanine-thymine) rich binding complementary sequence found on human chromosome 22 for germline integration, hoping for integration of raav into human chromosome 22.* In http://www.doddisnobelnomination1.blogspot.com,

"In order to generate recombinant adeno associated virus serotype 2 (raav2), the toll like receptor 9 (tlr9) ligand on the virus raav2 has to be deleted (mentioned in scientific papers) in order for the virus genome to evade the immune (antibody) response. My research discussion entails using recombinant adeno associated virus serotype 2 (raav2) which is antibody evasive- this is true due to the molecular modification done to the viral genome. It bypasses detection by the human immune system. The papers also points to the presence of a toll like receptor 2 (tlr2) ligand on

the virus, raav2 whose phenotypic expression appears on the capsid. One of my breakthroughs is that the tlr2 ligand on the virus too has to be deleted, to evade immune (antibody) response on the raav2 tlr2 capsid site.

Another molecular modification makes it more attractive to bind (integrate) to a particular chromosome (not chromosome 19). Also, another paper points to the presence of high gt (guanine-thymine) basepairs on human chromosome 22 which are good candidates for chromosomal targeting and integration of complementary ca (cytosine-adenine) base pairs on another molecule. Another breakthrough here is the realization that ca repeating motifs can be inserted in the virus in the empty space that was created by removing the tlr9 and tlr2 ligands as mentioned earlier.

A third molecular modification makes it carry the gene of interest (transgene)- to make raav2 truly recombinant the cap gene on the virus is removed and replaced by the transgene (gene of interest), typically chosen with care herein not to exceed 2.2-4.5 kb; preferably 2.2 kb".

CHAPTER 4

CONCLUSION

I. Conclusion

Recombinant adeno-associated virus serotype 2 (raav2) vectors carrying the pertinent transgene (not exceeding 2.2-4.5kb in size) can site-specifically integrate into human chromosome 19 or 22 in humans in an 'anti-body evasive' manner, leading to permanent genetic medicine with an estimated efficacy of 10-12 years per administered dose. This is optimized human gene therapy for ten human diseases. In addition, there is also the potential for development of a 'biosensor' to monitor the viral load in the human body from time to time.

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