Gene Therapy

Christopher D. Porada, Ph.D.

Why do we need gene therapy?
A treatment of this type promises to offer a precise/specific means of curing essentially any disease with a genetic defect as the root.

Current therapies are inadequate and do not CURE disease.

What is Gene Therapy?
The transfer of a normal functional copy of a gene into a somatic cell that has a mutation in its copy of the given gene.

Hemophilia

- Bleeding disorders caused by single gene defects
- First described by Abulcasim (the father of modern surgery) in 980 AD
- Queen Victoria made this the “royal disease”

What kind of diseases would be treatable by gene therapy?
- Diseases caused by a single faulty gene (over 4000 known).
- Candidate diseases include: Sickle cell anemia, Thalassemia, MPS, Gaucher’s, Hurler’s, Hemophilias

How Queen Victoria changed the course of European history
Comes in two varieties:
A: defect in Factor VIII
B: defect in Factor IX

Why is hemophilia A a good disease for gene therapy?
• Hemophilia A is caused by single gene defect in Factor VIII
• Expression need not be tissue-specific because product is secreted
• Very little of the factor is needed to produce a great improvement.
• Supraphysiologic levels well tolerated.

Hemophilia Phenotypes
• Severe: Less than 2% of normal levels of factor
• Moderate: 2-5% of normal levels
• Mild: 5% and above

Current Therapy for Hemophilia
• Factor injections; prophylactically and at bleed.
• Very expensive
• Lifelong treatment without cure
• Only available to 30% of the world’s hemophiliacs
• ~30% of patients develop inhibitory antibodies to FVIII

Three strategies for somatic cell gene therapy:
1. *Ex vivo* – cells removed from body, incubated with vector and gene-engineered cells returned to body.
2. *In situ* – vector is placed directly into the affected tissues.
3. *In vivo* – vector injected directly into the blood stream.
Example of *ex vivo* somatic cell gene therapy

Usually done with hematopoietic stem cells because they are easiest to remove and return.

Example of *in situ* somatic cell gene therapy

- Infusion of adenoviral vectors into the trachea and bronchi of cystic fibrosis patients.
- Injection of a tumor mass with a vector carrying the gene for a cytokine or toxin.
- Injection of a dystrophin gene directly into the muscle of muscular dystrophy patients.

### Stem Cell Transplantation

<table>
<thead>
<tr>
<th>Harvest HSC</th>
<th>Infuse normal donor cells</th>
<th>Radiation/Chemotherapy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donor</td>
<td></td>
<td>Patient</td>
</tr>
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</table>

Example of *in-vivo* somatic cell gene therapy

- Injection of liver-tropic AAV to treat hemophilia B.
- Safe *in vivo* injectable vectors must be developed.

### Stem Cell Gene Therapy

<table>
<thead>
<tr>
<th>Harvest HSC</th>
<th>Place gene in delivery vector</th>
<th>Expose cells to vector</th>
<th>Reinfuse corrected cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient</td>
<td></td>
<td>Patient is own donor!!</td>
<td></td>
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</tbody>
</table>

### Types of vectors

- **RNA viruses (Retroviruses)**
  1. Murine leukemia virus (MuLV)
  2. Human immunodeficiency viruses (HIV)
- **DNA viruses**
  1. Adenoviruses
  2. Adeno-associated viruses (AAV)
- **Non-viral vectors**
  1. Plasmids
  2. Episomal Vectors
Considerations

- What are the Target cells
- How much gene expression is needed
- Are target cells dividing
- Is persistent gene transfer needed
- Any inflammatory reaction to the vector
- Will repeat transfer be needed
- How large is the transgene

Why is genomic integration important?

Retroviruses

The first infectious agents implicated in tumors (Chicken sarcomas, identified by Rous, 1906)

Involved in infectious disease (HIV) and in cancer

Retroviruses (and transposable elements) appear to be part of every cell’s genome (bacteria, yeast, flies, fish, mice and humans), and comprise ~8% of the human genome!!!

Rudolf Jaenisch, 1976

Conducts first “gene transfer” study with retrovirus

Why would we want to use Retroviruses as vectors?

1. Have evolved over millennia to ensure efficient entry into target cells.
2. Wide host range (tropism can be modified)
3. Stably integrate into host cell genome [unique feature]
4. Long-term expression of transgene
5. Can easily modify genome for use as vector
6. “Non-pathogenic” in humans

A step in the right direction, but...

Since Jaenisch used wild-type virus, the virus could spread.

All mice developed viremia, many got leukemia (virus was MMLV)
How do we make a retrovirus safe for gene transfer?

Wild-type retroviral genome

Vector genome

As of 7/21/2014, there were 16,344 PubMed publications using retroviral vectors; 208 of these are clinical trials. There are currently 64 ongoing clinical trials using retroviral vectors. [ClinicalTrials.gov]

Are retroviral vectors effective?

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BBC News: 3rd October, 2002
Gene therapy trials halted

France and the United States have halted gene therapy trials after a child developed leukemia after undergoing treatment.

Disadvantages

1. Capacity for therapeutic genes is small (<8kb)
2. Inactivated by complement cascade
3. Infection limited to cells expressing receptor
4. Infectivity limited to dividing cells
5. Relatively low titters (10^6 particles/ml)
6. Randomly integrates into genome
   - Can’t predict level of transcription
   - Insertional mutagenesis

Integration Site Preference of MMLV
**Disadvantages**

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**Advantages of HIV-based vectors**

1. Can infect both dividing and non-dividing cells
   - Makes it ideal for HSC, neurons, and adult cell populations that aren’t actively cycling.
2. Can easily be pseudotyped to re-direct specificity (does this naturally)
3. 1st clinical trial using HIV vector in 2005; currently 22 ongoing clinical trials using HIV vectors

**How can we get the vector to infect non-dividing cells?**

1. Why won’t the vector get into non-dividing cells?
   - It doesn’t possess a protein for nuclear uptake
2. What can we do about this?
   - Make a vector from a virus that has such a protein

**Disadvantages of HIV-based vectors**

1. Capacity for therapeutic genes is still small
2. Much more complex genome than MMLV
3. Relatively low titers (10^6 particles/ml) unless use VSV pseudotype
4. Randomly integrates into genome
   - Can’t predict level of transcription
   - Insertional mutagenesis
4. HIV is a human pathogen

**Integration Sites of MMLV vs. HIV**

- **In Vivo Gene Delivery and Stable Transduction of Nondividing Cells by a Lentiviral Vector**
  - Science 272:263, 1996

- **Clinical Trials.gov**

- **Showed HIV could be used as the backbone for a retroviral vector system. Would efficiently transfer genes to cell cycle-arrested cells in culture and to post-mitotic neurons in vivo.**
Disadvantages with HIV

1. Capacity for therapeutic genes is still small
2. Relatively low titers (10^6 particles/ml) unless use VSV pseudotype and concentrate
3. Randomly integrates into genome
   - Can’t predict level of transcription
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4. HIV is a human pathogen

Adenoviral vectors

- Non-enveloped ds DNA, 36 kilobases
- Early proteins (E1A, E1B, E2,E3 and E4), late proteins (L1-L5)
- Causes a benign respiratory infections in human
- Serotypes 2 and 5 are commonly used as vectors

The HIV genome evolves ~1 million times faster than the human genome

Transmission and the formation of an HIV quasispecies

Transmission

Immediate descendants of one sequence predominates (usually)
~ All daughter viruses are genetically distinct

One error is incorporated into the HIV genome every 2,000-5,000 bases replicated (2-5 per genome)

Adenovirus

Advantages

- High transduction efficiency
- Insert size up to 8kb (gutted, up to 37kb)
- High viral titer (10^{10}-10^{13})
- Infects both replicating and differentiated cells
- Tropism to respiratory epithelial cells and liver cells

DNA Viruses as Vectors For Gene Transfer

Adenovirus Lifecycle
Adenovirus Disadvantages

- Receptor is ubiquitously expressed
- Transgene expression is transient (viral DNA does not integrate)
- In vivo delivery hampered by host immune response
- Viral proteins, and even empty capsid itself, can trigger robust inflammatory/immune response.

Modification of the tropism of adenovirus vector

- Adenovirus fiber binds to CAR (coxsakie and adenovirus receptor, CAR), receptor which is ubiquitous
- Modify the fiber protein (Curiel and Wickham)

How do we target adenoviral vectors?

Tumor-specific gene transfer via an adenoviral vector targeted to the pan-carcinoma antigen EpCAM

HJ Haisma, HM Pinedo, A van Rijswijk, I van der Meulen-Muileman, BA Sosnowski, W Ying, WV van Beusechem, BW Tillman, WR Gerritsen and DT Curiel

Volume 6, Number 8, Pages 1469-1474

Example of risks of Adenovirus-triggered immunity: Jesse Gelsinger

*First patient to die from gene therapy treatment.
Disease: liver enzyme deficiency (ornithine transcarbamylase, OTC) – controls ammonia metabolism
Goal: Use modified adenovirus to deliver vector to liver cells and express OTC.
Outcome:
- Vector not only delivered gene to liver but to other tissues.
- Triggered systemic inflammatory response.
- Patient acquired fever, coma, death.
Do the gutted adenoviral vectors work?

- Yes, and they appear to evade immune detection!

- Using a mouse model of DMD, Jeffrey Chamberlain and his group at the University of Washington in Seattle has now shown that gutted adenovirus carrying a mouse dystrophin gene seems to provoke very little immune response. Also, the dystrophin is effectively delivered to muscles, and in force measurement tests, treated muscles showed a lasting improvement in endurance compared to untreated muscles.

Advantages to AAV

- Efficient gene delivery to both dividing and non-dividing cells
- No viral genes to cause an undesirable immune response
- High levels of gene expression
- Site-specific integration into chromosome 19
- In vivo administration to patients
- Excellent stability allowing AAV vectors to be manufactured, stored, and handled like more traditional pharmaceutical products

Adeno-associated virus (AAV)

A very simple virus. It is a member of the parvoviridae family of small, non-enveloped viruses. AAV gets its name because it was discovered as a contaminant in a clinical sample of adenovirus over 40 years ago. Thus, it was given the name adeno-associated virus (AAV). AAV, however, shares none of its viral properties with adenovirus. In fact, their genes have nothing in common with each other. This is important because while adenovirus is pathogenic in humans, AAV is not.
Adenovirus-Associated Virus Vector–Mediated Gene Transfer in Hemophilia B

AAV Vectors

Disadvantages

• Insert size up to 4.5 kb
• Low transduction efficiency
• Low viral titer
• Not site-specific

Cons:

1. Strong immune reactions against viral proteins
2. Possibility of chromosomal insertion and proto-oncogene activation
3. Complicated synthesis process
4. Limitation on gene size
5. Toxicity, contamination with live virus

Viral Vectors

Pros:

1. High Transfection Efficiency
2. Natural Tropism (ability to infect different cells)
3. Evolved mechanisms for endosomal escape
4. Natural transportation mechanism of DNA into nucleus

Non-viral Methods of Gene Transfer

• Liposomes
• Naked DNA injection
• Electroporation
• Ballistic method

Non-viral DNA carriers

Liposomes: Spherical vesicles composed of synthetic lipid bilayers which mimic the structure of biological membranes and are thus able to traverse cell membranes.
Liposomes

- DNA coupled to a targeting ligand that binds to specific receptor
- Transferrin receptor expressed in many cell types, enriched in blood cells
- Can also be used with cell-specific receptors like asialoglycoprotein receptors, found on surface of hepatocytes
- Complexes could be infused into liver and taken up by specific cells

Marked enhancement in gene expression by targeting the human insulin receptor

Yun Zhang, Ruben J. Boado, William M. Pardridge*

This looks good on paper, but...

After endosome formation, most of the DNA is degraded and never gets to nucleus, so exerts no effect

Receptor-mediated endocytosis

The Future of Gene Therapy?

1=condensed DNA core
2=endosomalytic peptide
3=nuclear targeting peptide
4=hydrophilic shield
5=cleavable linker
6=targeting element
7=erythrocyte
**Physical methods**

<table>
<thead>
<tr>
<th>Advantage</th>
<th>Disadvantage</th>
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<td>Not limited by size or number of genes</td>
<td>Inefficient</td>
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Injection of naked DNA

Electroporation *in vivo* or *ex vivo*

Ballistic technology – the “gene gun”

**Naked DNA Injection**

- Was discovered by accident when naked DNA was used as a negative control in liposome expts
- Naked DNA (plasmid) can be directly injected into muscle cells
  - Injection has been used to target muscle cells in mice with *mdx* defect, model for Duchenne
- DNA believed to enter the cell through small lesions in cell membrane
- Not very efficient, but can result in prolonged low level expression *in vivo* if stably integrated.
- Useful in cells that don’t divide, like muscle cells

**The Helios Gene Gun by BioRad**

Targeted gene delivery is now a reality for *in vivo* research applications; DNA is attached to gold particles that are “fired” into the tissue on stream of helium.

**Ex vivo electroporation**

- cells obtained from skin biopsy
- genetically engineered cells are propagated
- gene introduced into cells by electroporation
- cells are reinjected

**Non-viral Methods**

**PROS:**

1. Low immunogenicity
2. Can be made to be non-toxic
3. Easy to synthesize: quality control for mass production
4. Potentially targetable
5. No limit on plasmid size
6. No integration: Can be administered as drugs
CONS:
1. Low transfection efficacy
2. Currently most vehicles are toxic at high doses
3. No natural tropism, endosomal escape, or nuclear transport mechanisms
4. No integration, so transient expression

Episomal Plasmid System
- Non-viral
- No integration
- Based on oriP/EBNA-1 from Epstein Barr Virus (EBV)
- Replicates along with cell genome while drug selection present
- Requires just a single transfection

Up to this point, every method we have discussed is simply “gene addition”

What if there were a way to actually change/correct the sequence of the genome?

EBNA1 regulates cellular gene expression by binding cellular promoters

- EBNA-1 is involved in EBV-induced tumors (lymphomas and several types of epithelial tumors).
- Canaan et al. showed it binds to ~100 cellular promoters.
- Unknown what effect this has in stability of cells modified with EBNA-1 containing episomes.
- Once drug selection is removed, episomes gradually lost

Genome-Editing Technologies
Zinc Finger Proteins

Originally discovered as TF in frog

Why would a gene therapist want/need this?

If you introduce a repair “template” DNA molecule with the ZFN, you get very efficient homologous recombination (HR), swapping donor sequence for the one ZFN cuts out of genome. Use of ZFN to introduce DSB at desired site increases HR efficiency by ~1,000 fold.

Zinc Finger Nucleases

What does this do for us?

Highly-specific Genomic Scissors

Can introduce double strand break (DSB) at any genomic location

Highly efficient endogenous human gene correction using designed zinc-finger nucleases.

So why hasn’t this technology solved all our problems?

- Very complicated to design; quite “hit or miss”, context-dependent activity
- Off-target effects can cause substantial toxicity (each finger targets only 3 nucleotides); need arrays
- Sangamo (very smart) owns all IP associated with ZFN and the algorithms to design them.


Are these perfect?

No; very large (1200aa), so hard to deliver to cells with high efficiency (don’t work well in viral vectors).

Given that they are bacterial in origin, they may trigger immune response in humans.

Still too early to know whether they are as site-specific as software predicts.

Are there other options?

Yes!

Transcription activator-like effector nucleases (TALENs)

Also fusion protein consisting of DNA-binding motif and FokI nuclease.

Originally identified in plant pathogens of the bacterial genus; activate genes in infected cells to support bacteria’s growth.

What is the “latest and greatest”?

CRISPR/Cas9 system

(clustered regularly interspaced short palindromic repeats)

Discovered in bacteria; represents an acquired immune system to protect bacteria from foreign invading DNA, i.e., viruses.

Consists of RNA molecule that recognizes specific DNA sequence, and Cas9 nuclease to cleave targeted DNA sequence.

Why are these any better?

Very easy to design/manufacture; simple cipher to determine which amino acids are needed to bind desired DNA sequence. Also cheap to produce.

The CRISPR/Cas9 system in action

[Diagram showing the CRISPR/Cas9 system]
What makes this unique/better?

DNA recognition portion is simple RNA molecule (20nt) that works via base-pairing; easy to design/manufacture via oligo synthesis. RNA-guided DNA endonuclease.

Can easily be delivered to human cells using transfection and viral vectors (lentiCRISPR from Addgene).

Very new (~1 year), but already taking field by storm (>750 Pubmed papers).

Where does this leave us?

- Each gene delivery system has its own advantages/disadvantages.
- Viral-based methods are still most efficient, but carry highest risk of tumorigenicity and immunogenicity.
- Non-viral are promising, but still need further tweaking.
- New genome-editing technology shows great promise.

Genome editing with Cas9 in adult mice corrects a disease mutation and phenotype

Hao Yiu1,2, Wen Xue1,2, Sidi Chen1, Roman I. Bogorad1, Eric Benedetti2, Markus Grompe2, Victor Kotliarsky3, Phillip A. Sharp1,4, Tyler Jacks5,6 & Daniel G. Anderson7,8,9

Delivered sgRNA and Cas9 in same plasmid via hydrodynamic injection (tail vein) along with 199bp donor DNA molecule, into mice with FAH deficiency (same single point mutation that causes hereditary tyrosinemia in people)

Generated FAH+ hepatocytes and corrected phenotype