Adeno-associated viral vectors and stereotaxic delivery

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Adeno-Associated Virus

- Parvovirus family
- Linear ssDNA of either polarity
- Infects dividing and non-dividing cells
- Persists in episomal state in host nucleus
- Can infect large number of cell types
- ~4.7 kb wild type genome
AAV Life Cycle
Packaging Components of rAAV

1. Transfer vector: Inverted Terminal Repeats (ITR) flanking gene of interest
2. AAV helper plasmid expressing rep and cap proteins
3. Adenovirus helper plasmid expresses adenoviral proteins necessary for packaging
AAV as a Viral Vector

**Strengths**
- Low immune response
- Infects dividing and non-dividing cells
- Non-integrating
- Persistent expression
- Serotypes differentially infect cell types
- Various routes of delivery
- High titers

**Weaknesses**
- Not ideal for dividing cells, DNA is lost through cell division
- Small genome allowing gene cassette of ~4.4kb
- Serotypes differentially infect cell types
AAV Serotypes

- AAV is subdivided into serotypes which exhibit different tissue tropism

- Serotypes are not tissue specific because they can transduce many different cell types

- Serotypes have higher affinities for certain cell types due to differences in capsid proteins
## AAV Serotypes Target Different Tissues

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Optimal Serotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>AAV8, AAV9, AAVDJ</td>
</tr>
<tr>
<td>Skeletal Muscle</td>
<td>AAV1, AAV6-9</td>
</tr>
<tr>
<td>CNS</td>
<td>AAV1, AAV2, AAV5, AAV8, AAV9, AAVDJ</td>
</tr>
<tr>
<td>Photoreceptor Cells</td>
<td>AAV5</td>
</tr>
<tr>
<td>Lung</td>
<td>AAV9</td>
</tr>
<tr>
<td>Heart</td>
<td>AAV8</td>
</tr>
<tr>
<td>Pancreas</td>
<td>AAV8</td>
</tr>
<tr>
<td>Kidneys</td>
<td>AAV2</td>
</tr>
</tbody>
</table>
rAAV Production

• Triple transfection
• Wait 48-72 hours
• Lyse cells by freeze/thaw or sonication
• CsCl gradient, iodixanol gradient or heparin column
• Dialyze fractions
• Titer by Taqman qPCR
• Titers >10^{12} vg/ml
Stereotaxic Delivery

- Good spatiotemporal control
- Any brain region or subpopulation of cells
- Inject at any postnatal date

Common Uses

- Fluorescent labeling of cell populations
- Neuronal track tracing
- Viral mediated gene knockdown or over-expression
- Cell specific targeting using cre transgenic mice
Stereotaxic injection protocol

- Anesthetize animal
- Secure rodent to stereotax, continue anesthesia
- Make incision and locate bregma
- Drill hole in skull at desired coordinate
- Load syringe with viral suspension
- Lower needle to proper coordinate
- Infuse viral particles at 0.1-0.5 ul/min
Intracerebral injection for protein over-expression

Serotype: AAV9
Protein Expressed: Girk1-2a-mCherry and Girk2-2a-GFP
Amount injected: $10^9$ vg
Manufacturer: VVC

Ford et al., unpublished
Cell specific targeting using cre transgenic

Serotype: AAVDJ
Protein expressed: ArchT-YFP
Amount injected: $10^9$ vg
Manufacturer: VVC

McGinley et al, unpublished
Intraventricular Injection

Serotype: AAV2  
Protein Expressed: GFP  
Amount injected: $10^8$ vg  
Manufacturer: UNC

Fu et al., Molecular Therapy, 2003
Intravenous Injection

Serotype: AAV9
Protein expressed: GFP
Amount injected: $4 \times 10^{11}$
Manufacturer: UPenn

Foust et al., Nature Biotechnology, 2009
Intranasal Injection

Serotype: AAV2
Protein Expressed: dsRed
Amount injected: $10^{10}$ vg
Manufacturer: VVC

Borisovska et al., unpublished
Serotype: AAV2
Proteins expressed: GFP and mCherry
Amount injected: \( \sim 10^8 \) vg
Manufacturer: UPenn

Deniz Kusefoglu et al., unpublished
Vollum Viral Core Services

- Free consultation

- Cloning, production and titering of AAV, lenti- and retro- viral particles

- Stereotaxic delivery of viral particles

- Contact: washbure@ohsu.edu
Biosafety Concerns of Viral Vectors—Focus Lentivirus

Debra Brickey PhD CBSP
May 5, 2011
Biosafety Risks of Lentivirus and Other Viral Vectors

- Recombinant DNA Advisory Committee (RAC)
- 2006 reviewed biosafety of Lentivirus System
Biosafety Risks of Lentivirus and Other Viral Vectors

- Potential for generation of replication competent virus
- Potential for oncogenesis

Risks are mitigated by the nature of the vector system and its safety features or exacerbated by the nature of the transgene insert encoded by the vector.
Criteria For Risk Assessment of Lentivirus Vectors

- The nature of the vector system and the potential for regeneration of replication competent virus from vector components.
- The nature of the transgene insert (e.g. known oncogenes or genes with high oncogenic potential merit special care)
- The vector titer and the total amount of vector.
- The inherent biological containment of the animal host, if relevant
- Negative RCL testing
Nature of the Vector System

- General containment considerations
  - HIV-1, HIV-2, and SIV
    - Wild-type: BSL2+ to BSL3
    - Vector Systems: BSL2 (rarely BSL2+)
  - FIV, BIV, and EIAV
    - Wild-type: BSL1
    - Vector Systems using VSV-G or other coat protein which broadens tropism to include mammalian: BSL2
Nature of the Vector System

- 2nd, 3rd, or 4th generation viral systems
  - Self-inactivation (SIN) decreases possibility of RCL and insertional mutagenesis
  - Deletion of viral proteins
    - 2nd generation (5 of 9 HIV-1 genes eliminated)
    - 3rd generation (only gag, pol, and rev remain and a chimeric 5’LTR)
    - 4th generation additional changes improving safety and viral production (many proprietary)
  - Separate packaging onto multiple plasmids (3 to 5)
  - Elimination of TAT expression that is required for viral replication
Nature of the Vector System

- **Concerns**
  - Mixing and matching systems, generations, and packaging cell lines
  - Experience of Lab personnel working with virus and at BSL-2
  - Special cases using 2\textsuperscript{nd} generation viral system
Nature of the Insert

- **Concerns**
  - Accidental exposure—Infectious and Integratable
    - Accidental Injection
    - Splash to mucous membranes, cuts
  - Non-Oncogene, non-viral inserts
    - Insertional Mutagenesis
  - Oncogene or Potential Oncogene and viral inserts
    - Insertional Mutagenesis
    - Integration and Expression of Oncogene
Titer, Amount, and Animal Use

Virus Titer and Total Amount of Virus

- Concerns
  - High titer
    - exposure more likely to result in infection and expression of insert
  - Large volume (greater than 10L)
    - exposure due to handling of large volumes during production
    - RCL event

Biological Containment of Animal Host

- Concerns (post-injection, injections at BSL2)
  - Non-permissive host—ABSL1
  - Special cases will increase standard ABSL containment—ABSL2
    - Host permissive for lentiviral replication
    - Host engrafted with human cells
Replication Competent Lentivirus Testing

- **RCL testing**
  - Required by FDA for use in human clinical trials
  - Not required for non-clinical, small volume, and low-risk genes
  - Based on risk assessment, the IBC may require RCL testing if larger volumes, high-risk genes, or vector system increase risk of RCL
    - 2006—Had to consider laboratory expertise for working with infectious lentivirus as positive control may increase risk to the investigator compared to the test material
    - P24 Elisa
Spills

- **Disinfectants**—
  - Choose disinfectant based on agent sensitivity and contact time
  - 10% Bleach or phenolic disinfectants for a VSV-G pseudotype

- **Inside Biosafety Cabinet (BSC)**
  - Spray with disinfectant and wipe up spill with paper towels
  - Dispose of paper towels to biohazard container (inside BSC)
  - Change to new gloves before continuing work
  - Disinfect thoroughly upon completion of work
Spills

- **Outside BSC**
  - Cover with paper towels, pour disinfectant over spill, allow a 10’ contact time, clean, and repeat (Dispose of materials in Biohazardous Waste Container)
  - Inform others in vicinity that a spill has occurred ASAP, do not leave spill unattended
  - Wash hands and replace any PPE that has been exposed
  - Report to Supervisor, consult BSO if exposure possible
  - Evaluate exposure risk
Exposures

- **IBC Research Accidents and Spills**
  - [Policy](http://www.ohsu.edu/xd/about/services/integrity/policies/policy-detail.cfm?policyid=1511935)

- **Splash to mucous membranes**
  - Rinse eyes, mouth and nose depending on exposure site with water for 10 minutes

- **Accidental Injection**
  - Encourage bleeding and wash site with soap and water

- **After Initial Response**
  - Report to Supervisor and BSO
  - Go to Employee Health or ED for further evaluation
  - Complete accident report: [https://ozone.ohsu.edu/wsirs/](https://ozone.ohsu.edu/wsirs/)
NIH rDNA Guidelines, BMBL, and Your Lab

- Guidelines used by IBC and the BSO to evaluate protocols and lab practices
  - NIH guidelines
  - BMBL 5\textsuperscript{th} edition

- Template for Laboratory Specific Lentiviral Manual
  - [http://www.ohsu.edu/xd/about/services/integrity/policies/policy-detail.cfm?policyid=1534831](http://www.ohsu.edu/xd/about/services/integrity/policies/policy-detail.cfm?policyid=1534831)
Risk Assessment of Procedures

- Work Practices that may require a Risk Assessment (not all inclusive)
  - Sterotactic injections
    - http://www.ohsu.edu/xd/about/services/integrity/policies/policy-detail.cfm?policyid=1908735
  - Animal injections occurring outside BSC
  - Work with live virus outside BSC
  - Use of sharps
Training

- BigBrain Courses (https://bigbrain.ohsu.edu/)
  - General and Laboratory Safety Course
  - Bloodborne Pathogen Training for OHSU Researchers
- Group training for BSL2 work practices by request
- DCM/DAR training for ABSL2 and animal injections
Good Luck and Be Safe With Your Research!
Choosing a Viral Vector System

Janet Douglas
20 years of Vector Development

Number of Gene Therapy Clinical Trials Approved Worldwide 1989 - 2010

The Journal of Gene Medicine, © 2011 John Wiley and Sons Ltd

www.wiley.co.uk/genmed/clinical
Vectors Used in Gene Therapy Clinical Trials

- Adenovirus 24.1% (n=410)
- Retrovirus 20.8% (n=354)
- Naked/Plasmid DNA 18.7% (n=319)
- Vaccinia virus 8% (n=137)
- Lipofection 6.4% (n=109)
- Poxvirus 5.5% (n=94)
- Adeno-associated virus 4.8% (n=81)
- Herpes simplex virus 3.3% (n=57)
- Lentivirus 2.2% (n=38)
- Other categories 5.2% (n=89)
- Unknown 3.2% (n=55)
Considerations

- What are your target cells?
- Are they dividing or non-dividing?
- Do you want transient or long-term expression?
- Will an immune response to the vector affect your results?
- Is gene expression going to be evaluated in vitro or in vivo?
- Do you have access to and training for a BSL-2 lab?

<table>
<thead>
<tr>
<th></th>
<th>Adenovirus</th>
<th>AAV</th>
<th>Lentivirus</th>
<th>Retrovirus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gene Expression</td>
<td>Transient</td>
<td>Transient or Stable</td>
<td>Transient or Stable</td>
<td>Stable</td>
</tr>
<tr>
<td>Infect Dividing Cells</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Infect Non-Dividing Cells</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Integration into Target Cell Genome</td>
<td>No</td>
<td>No*</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Immune Response in Target Cells</td>
<td>High</td>
<td>Very Low</td>
<td>Low</td>
<td>Moderate</td>
</tr>
<tr>
<td>Relative Viral Titer</td>
<td>XXXX</td>
<td>XXX</td>
<td>XXX</td>
<td>XX</td>
</tr>
<tr>
<td>Relative Transduction Efficiency</td>
<td>XXXX</td>
<td>XXX</td>
<td>XXX</td>
<td>XX</td>
</tr>
</tbody>
</table>

*Native AAV will integrate, but recombinant AAV rarely does.
Tropism

- Not all viral vectors are “designed” to infect all cell types... tropism of virus = tropism of vector
- Some are more versatile than others
  - Retroviruses/lentiviruses can be pseudotyped with a wide variety of envelope proteins to broaden tropism
    - Mouse specific... ecotropic MuLV envelope
    - Mouse and human... amphototropic MuLV envelope
    - Most vertebrates... VSV-G
  - AAV inherently broad tropism, but can increase with different serotypes
  - Adenovirus pretty broad, but needs CAR (receptor)
    - Low in human hematopoietic cells & mouse cells in general
    - Can swap fiber from other serotype
Promoter issues

• Tissue/cell type specific expression
  – ie. Liver, Endothelial cells, Cancer cells
• Inducible expression for toxic gene products
  – Tetracycline-operator
• High expression levels
  – Viral promoters (CMV; RSV; SV40)
    • Downside is they may be targeted by anti-viral mechanisms in vivo
• Consistent expression levels
  – Eukaryotic promoters (PGK; EF-1α)
• Multiple genes
  – Multiple promoters
  – Internal ribosome entry site (ires)
  – Vector size restraints and variable expression
  – Self-cleaving 2A peptide fused to transgene product
    • Especially useful for AAV (small packaging size)
<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Adenovirus</th>
<th>Adeno-associated virus</th>
<th>Alphavirus</th>
<th>Herpesvirus</th>
<th>Retrovirus / Lentivirus</th>
<th>Vaccinia virus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genome</td>
<td>dsDNA</td>
<td>ssDNA</td>
<td>ssRNA (+)</td>
<td>dsDNA</td>
<td>ssRNA (+)</td>
<td>dsDNA</td>
</tr>
<tr>
<td>Capsid</td>
<td>Icosahedral</td>
<td>Icosahedral</td>
<td>Icosahedral</td>
<td>Icosahedral</td>
<td>Icosahedral</td>
<td>Complex</td>
</tr>
<tr>
<td>Coat</td>
<td>Naked</td>
<td>Naked</td>
<td>Enveloped</td>
<td>Enveloped</td>
<td>Enveloped</td>
<td>Enveloped</td>
</tr>
<tr>
<td>Virion polymerase</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td>Virion diameter</td>
<td>70 - 90 nm</td>
<td>18 - 26 nm</td>
<td>60 - 70 nm</td>
<td>150 - 200 nm</td>
<td>80 - 130 nm</td>
<td>170 - 200 X 300 - 450 nm</td>
</tr>
<tr>
<td>Genome size</td>
<td>39 - 38 kb</td>
<td>5 kb</td>
<td>12 kb</td>
<td>120 - 200 kb</td>
<td>3 - 9 kb</td>
<td>130 - 280 kb</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Family</th>
<th>Adenoviridae</th>
<th>Paroviridae</th>
<th>Togaviridae</th>
<th>Herpesviridae</th>
<th>Retroviridae</th>
<th>Poxviridae</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infection / tropism</td>
<td>Dividing and non-diving cells</td>
<td>Dividing and non-diving cells</td>
<td>Dividing and non-diving cells</td>
<td>Dividing and non-diving cells</td>
<td>Dividing cells*</td>
<td>Dividing and non-diving cells</td>
</tr>
<tr>
<td>Host genome interaction</td>
<td>Non-integrating</td>
<td>Non-integrating*</td>
<td>Non-integrating</td>
<td>Non-integrating</td>
<td>Integrating</td>
<td>Non-integrating</td>
</tr>
<tr>
<td>Transgene expression</td>
<td>Transient</td>
<td>Potential long lasting</td>
<td>Transient</td>
<td>Potential long lasting</td>
<td>Long lasting</td>
<td>Transient</td>
</tr>
<tr>
<td>Packaging capacity</td>
<td>7.5 kb</td>
<td>4.5 kb</td>
<td>7.5 kb</td>
<td>&gt; 30 kb</td>
<td>8 kb</td>
<td>25 kb</td>
</tr>
</tbody>
</table>
Recombinant Viral Vector Systems

• Vector has characteristics of parent virus
  – Capsid/Env dictates tropism
  – Viral genome maintenance dictates transient or long-term expression
  – Viral genome size dictates packaging size
  – Viral release determines vector preparation
    • Cell-associated and/or supernatant

• Safety Features
  – Packaging cell lines & helper constructs
Adenoviral particle

Receptor Binding
Recombinant Adenovirus

- Ad5-based vectors require Coxsackie-adenovirus receptor (CAR) on target cells
  - Low in human hematopoietic cells & mouse cells in general
- Transient gene expression
- High immune response
- High titers & transduction efficiency
Recombinant Adeno-associated virus (AAV)

- Broad tropism
- Low immunogenicity
- Helper virus free systems
- BSL-1
- Disadvantage is small packaging size 4.5kb
Retroviral virion components

- **Gag** = structural proteins
  - Matrix
  - Capsid
  - Nucleocapsid
- **pol** = enzymes
  - Protease
  - Reverse Transcriptase
  - Integrase
- **Env** = surface glycoproteins
- **Genome** = RNA
Recombinant Retroviruses

- Can be pseudotyped with various env proteins to broaden tropism
- Stable packaging cells
- Long-term gene expression through integration
  - Downside is insertional mutagenesis
- Disadvantage is only infects dividing cells
Retroviral vector design

The Helper:

Delete packaging signal

The Vector:

Maintain packaging signal
The Problem with recombination

- RCR-replication competent retrovirus
The Solution

Split the Helper genome

Or...
Lentiviral vectors

- Long-term gene expression like simple retrovirus
- Advantage is ability to infect non-dividing cells

- HIV-based systems
- Non-human based systems
  - Feline Immunodeficiency virus (FIV)
  - Equine infectious anemia virus (EIAV)
  - Simian immunodeficiency virus (SIV)
Evolution of Lentiviral Vector Development
## Overview

### The main groups of viral vectors

<table>
<thead>
<tr>
<th>Vector</th>
<th>Genetic material</th>
<th>Packaging capacity</th>
<th>Tropism</th>
<th>Inflammatory potential</th>
<th>Vector genome forms</th>
<th>Main limitations</th>
<th>Main advantages</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Enveloped</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Retrovirus</td>
<td>RNA</td>
<td>8 kb</td>
<td>Dividing cells only</td>
<td>Low</td>
<td>Integrated</td>
<td>Only transduces dividing cells; integration might induce oncogenesis in some applications</td>
<td>Persistent gene transfer in dividing cells</td>
</tr>
<tr>
<td>Lentivirus</td>
<td>RNA</td>
<td>8 kb</td>
<td>Broad</td>
<td>Low</td>
<td>Integrated</td>
<td>Integration might induce oncogenesis in some applications</td>
<td>Persistent gene transfer in most tissues</td>
</tr>
<tr>
<td><strong>Non-enveloped</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AAV</td>
<td>ssDNA</td>
<td>&lt;5 kb</td>
<td>Broad, with the possible exception of haematopoietic cells</td>
<td>Low</td>
<td>Episomal (&gt;90%) Integrated (&lt;10%)</td>
<td>Small packaging capacity</td>
<td>Non-inflammatory; non-pathogenic</td>
</tr>
<tr>
<td>Adenovirus</td>
<td>dsDNA</td>
<td>8 kb* 30 kb*</td>
<td>Broad</td>
<td>High</td>
<td>Episomal</td>
<td>Capsid mediates a potent inflammatory response</td>
<td>Extremely efficient transduction of most tissues</td>
</tr>
</tbody>
</table>
Lentiviral Vectors: design, production, and titration

ONPRC Lentiviral Vector Core
Molecular and Cellular Biology Core
Greg Dissen
2\textsuperscript{nd} and 3\textsuperscript{rd} generation viral vectors

1. Viral backbone was stripped to allow room for transgenes

2. Development of the Self-Inactivating (SIN) vector
2. Modification of 3’LTR “Self Inactivating”

Lentiviral Vector System

-456

U3

AP-1

NF-AT?

EcoRV

NF-κB

TATA

-418

U5

R

NF-Ets

TCF-1α

SP1

-18

PvuII

400 bp Deletion

ΔU3

R

U5

Wild-Type HIV 3’ LTR

pHR’ SIN-18
Lentiviral Vector System (3rd generation)

Integration into the Host Genome

No LTR promoter interference

Self Inactivation
HIV provirus

Lentiviral Vector Systems

2nd Generation vector

Requires Tat for production

3rd Generation vector

Constitutive promoter
RSV or CMV

Tat is not required
Lentiviral Vector Generations

HIV provirus:

1\textsuperscript{st} Generation:
- HIV-1 core proteins
- Enzymes and Accessory factors
- From separate plasmid and env plasmid

2\textsuperscript{nd} Generation:
- Packaging reduced
- gag, pol, tat, rev
- And env plasmid

3\textsuperscript{rd} Generation:
- Requirement for tat eliminated, rev moved to separate plasmid

FIRST GENERATION (1996)
- pLV
- pMD.G

SECOND GENERATION (1997)
- pLV
- pMD.G

THIRD GENERATION (1998)
- pLV
- pMD.G
- pRSV-Rev or pLP2
- pMDLgpRRE or pLP1
Packaging plasmids

4th generation?

Note: The components that make up the Lenti-X family of products are designed to work together as a system. Substituting components from other manufacturers or that users have developed in-house, may affect performance and or safety. We recommend that you utilize our complete system, however, if you do decide to use components other than those developed by Clontech, please carefully consider performance and safety implications.

Figure 1: Clontech's Lenti-X HT Packaging System consists of 6 separate components (Panel A), mixed in proprietary proportions for optimized packaging activity. The separation of the gag, pol, and env genes effectively reduces the incidence of RCL (Wu et al. 2000). Other 3rd generation systems (Panel B) which do not contain separate gag and pol sequences have higher RCL-generating potential. High levels of expression of essential viral components are driven by the Tet-Offs and Tat transactivators, resulting in high titers of virus. The pol gene is fused to vpr to ensure transport of the reverse transcriptase/integrate protein into the recombinant viral particle. Not all vector elements are shown.
Rev is essential for viral replication
Binds mRNAs removing them from splicesome = full-length and partially spliced

Both cPPT and wPRE increase
Transduction efficiency and transgene expression
Lentiviral Vector Systems

Reporter Vector

5’LTR  Ψ  SD  RRE  cPPT  hCMV-P  eGFP  wPRE  SIN3’LTR

RNA Polymerase II  Reporter

Constitutive:
CMV
SV40
hEFp
PGK

Tissue Specific
Lentiviral Vector System (3rd generation)

New components:

Internal Ribosome Entry Site:

2nd promoter

Allows the production of two proteins from one mRNA. A Bicistronic RNA.

Allows the production of two mRNAs from one vector.
Lentiviral Vector System (3rd generation)

New components:

Multiple Cloning Site for transgenes to be expressed:
A heterologous intron had been found to increase expression of transgenes in transgenic mice.

Insertion of a heterologous Intron

Rat insulin II intron A
Lentiviral Vector System (3rd generation)

CMV → IRES

NGF

Intron

IRES

Small peptide

Ligand is produced and expression is enhanced from vector containing heterologous intron
Lentiviral Vector System (3rd generation)

CMVp, Intron, Jag, IRES, eGFP

1. CMVp, Intron, Jag, IRES, eGFP, ΔU3, R, U5
2. CMVp, Intron, Jag, IRES, eGFP, ΔU3, R, U5
3. CMVp, Intron, Jag, IRES, eGFP, ΔU3, R, U5
4. CMVp, Intron, Jag, IRES, eGFP, ΔU3, R, U5
5. CMVp, Intron, Jag, IRES, eGFP, ΔU3, R, U5

Jag

140 kD

GAPDH

42 kD

GFP

27 kD
Lentiviral Vector System (3rd generation)

CMVp Intron Jag IRES eGFP

DU3 R U5

Jag

1 2 3 4 5

GAPDH

Titer Values (TU/mL)

<table>
<thead>
<tr>
<th></th>
<th>Real-time</th>
<th>GFP</th>
<th>Mean Fluorescence</th>
</tr>
</thead>
<tbody>
<tr>
<td>LV1</td>
<td>1.50E+07</td>
<td>7.10E+06</td>
<td>35.21 RFU</td>
</tr>
<tr>
<td>LV2</td>
<td>1.36E+07</td>
<td>3.34E+06</td>
<td>24.31 RFU</td>
</tr>
<tr>
<td>LV3</td>
<td>1.55E+07</td>
<td>bkg</td>
<td>bkg</td>
</tr>
<tr>
<td>LV4</td>
<td>1.10E+07</td>
<td>1.20E+07</td>
<td>54.39 RFU</td>
</tr>
<tr>
<td>LV5</td>
<td>2.26E+07</td>
<td>1.42E+07</td>
<td>66.26 RFU</td>
</tr>
</tbody>
</table>

GFP
Infection of Hib5 (6 Well plate, 200,000 cells/well)
miR30=
Retinoblastoma (Rb)
Targeting sequence:
AGCAGTTTCGATATCTACTGAAA

pPRIME-CMV-GFP
miR30-shRNA

Pol II driven shRNA was more active than the Pol III construct.

Stegmeier, 2005
Lentiviral Vector System: Gene Suppression

New components:

486 bp

U6-siRNA MCS Cassette

Bgl II  Kpn I  U6 promoter  *  Apa I  Xho I  Sal I  Cla I  Hind III  Eco RV  *  Eco RI  Sac II  Bst XI  Sac I  T3  Bgl II
Lentiviral Vector System: Gene Suppression

New components:

U6-siRNA MCS Cassette

486 bp
Virus Production

Day 1

1. **Cells:** human Embryonic Kidney 293T/17
   Cells have been transformed with temperature sensitive large T antigen
   Strain was selected specifically for its high transfectability

2. Cells are grown in antibiotic free conditions DMEM (1.5 g/l Na Bicarbonate), 4.5 g/l Glucose, Defined fetal bovine serum, 10% CO2
   Advantage to antibiotic free medium = immediately know when there is a problem/contamination

3. Cells are plated to achieve 70 confluency in 10 cm dishes that have been coated with poly-L-lysine (6 to 11 x 10^6 cells/dish)
Lentiviral Vector System

Transgene

- pLV 7,438 bp
  - 5'LTR RRE cPPT
  - hCMV-P
  - eGFP
  - WPRE
  - SIN3' LTR
- pMDLgpRRE GAG
  - 8,895 bp
  - hCMV-P
  - Pol
  - RRE
  - Poly A
- hβGlobin
  - IVS2
  - hCMV-P
  - RRE
  - Poly A

Packaging

- pRSV-Rev 4,174 bp
  - Pol
  - RSV
  - REV
  - Poly A

Envelope

- pMD.G 6,010 bp
  - hCMV-P
  - hβ Globin
  - IVS2
  - VSV G
  - Poly A

Transfection

- pLV
  - CaPO₄
- pMDLgp.RRE
  - CaPO₄
- pRSV-Rev
  - CaPO₄
- pMD.G

293T Cells
Lentiviral Vector System

**Transgene**
- pLV 7,438 bp
  - 5'LTR RRE cPPT
  - hCMV-P
  - eGFP
  - WPRE
  - SIN3' LTR

**Packaging**
- pMDLgpRRE 8,895 bp
  - hCMV-P
  - GAG
  - Pol
  - Poly A
  - RRE

**Envelope**
- pRSV-Rev 4,174 bp
  - RSV
  - REV
  - Poly A

**Transfection**
- pLV
- pMDLgp.RRE
- pRSV-Rev
- pMD.G
  - hβ Globin IVS2
  - VSV G

Transfected 293T cells
Control 0.29%

HEPES - Transfection 99.18%

BES - Transfection 99.8%
FACs Titer:

- pMDLpg.RRE
- pRSV-Rev
- pMD.G

Transfection

Conditioned Medium

FACs detects infected cells, expressed as percentage of total.
Virus Production

Titer Analysis Possibilities:

FACS for gene expression product:
- Dependent on Promoter activity
- Constitutive promoter = useful Titers that predict infection rate
- Tissue specific promoters might not give useful titers

Real-Time PCR for integrated viral DNA in host genome
- Dependent on infection and integration into the host genome
- Real-Time PCR Titers predict infection rate

Reverse transcription Real-Time PCR for viral RNA
- Dependent only on the presence of the viral RNA
- Does not predict infection rate of the viral particles
Acknowledgements

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Director, Lentiviral Services
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Fluorescence in 293-T embryonic kidney cells

- CMV
- Short GnRH
- Long GnRH

% fluorescence vs. viral prep (µl)
Fluorescence in GT1-7 neuronal cells

- CMV
- short GnRH
- long GnRH

% fluorescence vs. viral prep (µl)
Replication Competent Lentivirus (RCL)

3rd Generation Lentiviral Vector

Replication competent LTR
Gag, pol, rev, env, tat

Source:
- Carry over from packaging or Envelope plasmids
- Or
- Endogenous viruses

Wild-Type Virus
Replication Competent Lentivirus (RCL)

Protocol:

1. Infect 1 million SupT-1 cells with 5 million viral TUs

2. Pass the cells 3 times over 2-3 weeks

3. Test the medium for p24 protein with ELISA kit (commercial)
The Molecular Virology Support Core: Adenoviral Vectors and Beyond

Christoph A. Kahl, Ph.D.
Viral Vector Workshop, May 5th, 2011
Outline

1) Overview of the MVSC

2) Adenoviral Vectors

3) Expertise and Services
Overview

• What does the MVSC offer?
  – Comprehensive and broad array of virology services
  – Broad virology expertise, particularly in non-human primate (NHP) virology
    • Viruses and virus-derived products (viral vectors, antigens, wild-type strains)
    • Viral diagnostics (tissue viral loads)
    • Virus serology (antibodies)

• Virology Core Director
  • Christoph Kahl, newly recruited in March 2010
  • Background
    – Gene therapy and recombinant vaccines
    – Lentiviral and adenoviral vectors

• How to find the MVSC:
  – Physical location:
    • OHSU West Campus (ONPRC), Research Building: Room 46 (lab), Room 163 (office)
  – Online:
    • OHSU Website: Research > Research Cores & Shared Resources > Virology
    • ONPRC Website: Research Services > Research Support Cores > Virology
    • VGTI Website: VGTI > Core Services > Virology Core
    • “eagle-i consortium” Website
Adenoviral Vectors

• General features:
  – Replication-defective (unless in complementing cells)
  – Large transgene carrying capacity (~7-8 kb in ΔE1/E3 vector)
  – High titer production possible (up to $10^{13}$ vp at research scale)

• Common uses:
  – Transient gene expression
  – *in vitro* protein expression studies (high level expression)
  – *in vivo* vaccination and cancer therapy
    • Strong innate immune activation and immunogenicity

• Ad5
  – Most common vector serotype
  – Very broad tropism
    • Dividing and non-dividing cells
    • For transduction of HSC, DC, Synovio, VEC, smooth muscle need other serotype fibers
    • Targets liver upon system injection (can ablate by triple mutation in CAR, RGD, and KKTK)
### Adenoviral Vectors

<table>
<thead>
<tr>
<th>Species</th>
<th>Serotype</th>
<th>Receptor(s)</th>
<th>Tropism:</th>
<th>Seroprevalence (%)</th>
<th>Fibre shaft repeats</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>12, 18, 31</td>
<td>CAR, fIX, fX</td>
<td>Cryptic (enteric, respiratory)</td>
<td>35–70</td>
<td>23</td>
</tr>
<tr>
<td>B1</td>
<td>3, 7, 16, 21, 50</td>
<td>CD46, ‘X’, fX CD80, CD86</td>
<td>Respiratory, ocular</td>
<td>2–15 (Ad16, 21, 50) 35–70 (Ad3, 7)</td>
<td>6</td>
</tr>
<tr>
<td>B2</td>
<td>11, 14, 34, 35</td>
<td>CD46, ‘X’, fX CD80, CD86</td>
<td>Renal, ocular, respiratory</td>
<td>1–3 (Ad11, 34, 35) 18 (Ad14)</td>
<td>6</td>
</tr>
<tr>
<td>C</td>
<td>1, 2, 5, 6</td>
<td>CAR, fIX, fX, Lf, DPPC, VCAM-1, HS, MHC1-α2</td>
<td>Respiratory, ocular lymphoid</td>
<td>40–80</td>
<td>22</td>
</tr>
<tr>
<td>E</td>
<td>4</td>
<td>CAR</td>
<td>Ocular, respiratory</td>
<td>45</td>
<td>12</td>
</tr>
<tr>
<td>F</td>
<td>40, 41</td>
<td>CAR</td>
<td>Enteric</td>
<td>41 (together)*</td>
<td>12 (short fibre) and 21/22 (long fibre)</td>
</tr>
<tr>
<td>G</td>
<td>52</td>
<td>ND</td>
<td>Enteric</td>
<td>ND</td>
<td>9 or 17</td>
</tr>
</tbody>
</table>

ND: not determined.

*Serotypes 40 and 41 are very closely related antigenically.

Adenoviral Vectors

• **Essential steps:**

1. **Vector design**
   - Clone transgene or expression cassette into appropriate adenovirus shuttle plasmid
   - Need serotype-specific shuttle and vector genome backbone plasmids

2. **Vector generation**
   - Homologous recombination in mammalian cells
     - Adψ5 system in 293 CRE cells (Hardy et al JVI 1997, Ad5 ΔE1/E3)
   - Homologous recombination in bacteria
     - Shuttle plasmid with homologous end sequences (e.g. AdEasy)

3. **Vector amplification and production**
   - Vector amplification and passaging
   - Seed stock generation
   - Production of purified high-titer vector stock
     - Scaleable
     - Different purification methods
     - Can remake new vector stock from prior vector prep or lysate (no need for new transfection)
Mammalian Recombination

Bacterial Recombination

Hardy et al., JVI 1997

Coloncancer.org
Adenoviral Vectors

- **QC Testing Methods:**
  - Vector stock titration
    - **Physical titer = total # of viral particles (vp)**
      - DNA dye detection assay
      - Spectrophotometric reading of absorbance at 260 nm
    - **Infectious titer = biologically active virus only**
      - Limiting dilution assays (TCID$_{50}$, plaque forming unit assay)
      - Immunofluorescence assays (focus-forming unit assay)
  - Vector Function and Integrity
    - **Transgene expression**
      - Appropriate assay for transgene (Western Blot, ELISA, IFA)
    - **E1-region integrity**
      - Transgene and E1-spanning PCR
      - Transgene cassette sequencing
    - **Vector genome integrity**
      - Restriction enzyme analysis
  - **Biosafety**
    - Replication-competent adenovirus (RCA) assay
Expertise and Services

• Viral stocks, vectors and antigens
  – Adenovirus
    • Adenoviral Vectors:
      – Adψ5 system (ΔE1/E3)
      – Inducible CMV Promoter by using Ad(Transgene) vector + Ad(Tet TA) vector
      – Other Ad vector systems and serotypes upon request
    • Adenovirus Antigen
  – Adeno-associated Virus (AAV) *In planning*
    • AAV Vectors:
      – MVSC planning to offer custom AAV production for NHP AAV studies
      – Targeted gene expression in different tissues *in vivo*
Expertise and Services

- Viral stocks, vectors and antigens (continued)
  - RhCMV Vectors
    - WT virus and vector
      - Production, titration, plaque purification, growth curves
      - Persistent gene expression (replicating vector)
      - Immunogenic in vivo (SIV T cell vaccines)
    - RhCMV antigen
  - Lentivirus
    - HIV, SIV, and SHIV:
      - WT Stock production and titration
      - Virus susceptibility assays
    - Lentiviral Vectors:
      - Currently provided by the MCB Core at ONPRC (Eliot Spindel)
      - Contact Greg Dissen if interested
  - Vaccinia Virus
    - MVA Vectors:
      - Virus Stock production and titration
Expertise and Services

• Virus Diagnostics (Serology)
  – Qualitative
    • Co-Culture assays for SIV, RhCMV
  – Quantitative
    • qPCR assays for SIV, RhCMV, VZV/SVV (future)
  – Viral Antibodies
    • ELISA for SIV, RhCMV, VZV/SVV (future)
    • Consult with ONPRC SPF lab for RM screening

• Resources
  – Critical reagents (cell lines, virus strains, antisera etc.)

• Consulting and Research Support
  – Virology techniques and procedures
  – Virology studies in NHP
  – Working with viral biohazardous agents
  – IBC protocols
Expertise and Services

• Information needed from user:

1. Initial service request

2. IBC and IACUC approval for infectious agent

3. Alias account information for billing
Questions?