Monitoring Gene Transfer and Expression

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Hematopoietic Stem Cells
(blood-forming stem cells)

- Best characterized adult stem cells are hematopoietic stem cells (HSCs)
- Continuous propagation of human HSCs in culture has not yet been realized
- Lack of understanding of the genetic programs governing stem cell self-renewal and lineage determination

Target cells for gene transfer: hematopoietic stem cells.

The hematopoietic stem cell is capable of self-renewal and differentiation into all of the blood cell lineages.

References:


Gene Transfer into Hematopoietic Stem Cells

• Decipher mechanisms regulating hematopoietic stem cell self-renewal and lineage determination
• Understand leukemia and other blood diseases
• Enrich target cell populations for gene and cell therapies

Our motivation for transferring genes into hematopoietic stem cells is to set up experimental systems that will allow us to study regulatory mechanisms of blood cell development, and to model potential gene and cell therapies.

References:


Overview

➢ Enrichment Strategies for HSCs
  • Murine models: bone marrow, fetal liver
  • Human: bone marrow, mobilized peripheral blood, cord blood

➢ Method of Gene Transfer
  • Oncoretroviral vectors (MSCV)
  • Lentiviral vectors (HUMV)

➢ Monitoring Expression of Transgenes/Reporter Genes
  • Antibiotic resistance, enzymatic activity, cell surface protein
  • Fluorescent protein (FP) genes

➢ Lab Preview on Multiple Gene Expression
  • Simultaneous analysis of four fluorescent proteins
Enrichment Strategies for HSCs

Why is it necessary to enrich for hematopoietic stem cells?
The hematopoietic stem cell is the precursor to all of the mature blood cell types. Its frequency is approximately 1/10,000 to 1/100,000 of total bone marrow cells. Most hematopoietic stem cells are quiescent or slowly proliferating.

Different strategies are used to enrich for hematopoietic stem cells. In practice, hematopoietic stem cell-enriched populations contain stem cells and more differentiated progenitor cells.
Negative enrichment strategies deplete mature cells.

Positive enrichment strategies enrich for hematopoietic stem/progenitor cells.

Other methods exploit characteristics attributed to hematopoietic stem/progenitor cells. Some are based on efflux properties of the vital dyes Rhodamine 123 and Hoechst 33342.
Examples of the “Side Population” (SP) assay performed on mouse bone marrow cells and human cord blood cells.

The SP assay identifies hematopoietic stem/progenitor cells.

Reference:
Our preferred method of gene transfer is the retrovirus-mediated gene delivery system.
Retroviruses

- Retroviruses are enveloped viruses with a diploid RNA genome
- Obligate step in the life cycle: replicate through a DNA intermediate which is stably integrated into host chromosomal DNA
- Capacity to efficiently introduce and stably express their genomes in mammalian cells
Retroviruses are used as vehicles in gene transfer: oncoretroviral vectors, lentiviral vectors and foamy virus vectors. Only the former two types of vectors will be discussed.

Oncoretroviruses require dissolution of the nuclear membrane in order to translocate to the nucleus and integrate into host chromosomal DNA. Therefore, they can only infect dividing cells.

Lentiviruses (including the HIV-1 virus) have evolved a nuclear-import machinery that allows them to infect non-dividing as well as dividing cells.
An important consideration in the derivation of oncoretroviral vectors is to ensure that only intended target cells are gene-modified, for safety reasons. Replication-defective oncoretroviral vectors have been developed for this purpose.

The basic strategy is to separate the viral genome into a two-component system: viral elements essential for traversal through the retrovirus life cycle, and viral genes coding for the packaging proteins.

Reference:

Production of Oncoretroviral Vector Particles

Method to introduce transgenes (and reporter genes) into target cells using oncoretroviral vectors:
1. Engineer a packaging cell line producing viral packaging proteins.
2. Engineer an oncoretroviral vector containing viral essential elements, a transgene and a reporter gene.

Target cell expresses the transgene and reporter gene, but does not synthesize any packaging proteins. As a result, it cannot produce any infectious viral particles.
Method to evaluate transgene expression in target cells (mouse hematopoietic stem cells): mouse bone marrow transplantation model.

References:


Method to evaluate transgene expression in target cells (candidate human hematopoietic stem cells): experimental SCID-repopulating cell assay.

This assay is used as a surrogate assay (instead of conducting human clinical trials) to evaluate the in vivo repopulating potential of candidate human hematopoietic stem cells, referred to as SRCs (SCID-Repopulating Cells).

References:


Example of transgene expression in progenies of SRCS (candidate human hematopoietic stem cells) transduced with an oncoretroviral vector.

Successful transduction required 4 days because oncoretroviruses require cell division for nuclear translocation and integration into genomic DNA (chromosomes) of target cells. Recall that the majority of hematopoietic stem cells are quiescent. Upon stimulation by cytokines, a lag period of about 3 days precedes entry into cell cycle and traversal through S-phase. Culturing hematopoietic stem cells for 4 days risks losing their stem cell characteristics (such as the self-renewal capability).

This limitation prompted development of lentiviral vectors based on the HIV-1 virus.

Reference:

Limitations of Oncoretroviral Vectors for Stem Cell Gene Transfer

- Cell division is required for integration of vector DNA (majority of hematopoietic stem cells are not rapidly dividing - G0/G1)
- Target cells must express appropriate receptors
- Vector particles are relatively unstable (difficult to concentrate)
- Vector RNA size constraints (will accommodate ~ 6 kb)

The HIV-1 virus can infect non-dividing as well as dividing cells. However, the HIV-1 virus targets CD4+ T cells and monocytes.

To expand the host range of lentiviral vectors, the HIV-1 envelope protein is replaced with a more versatile envelope protein.
Host Range and Target Cell Type

- VSV-G-pseudotyped lentiviral vectors can transduce virtually all types of cells
  
  Glycoprotein G of vesicular stomatitis virus (VSV-G) binds to a phosphatidyl serine component of the lipid bilayer present in the cell membrane of most eukaryotic cells

- Differentiated cells and tissues as diverse as neurons, liver, muscle, and retina

- Undifferentiated stem cells

VSV-G greatly expands the host range of lentiviral vectors.
Derivation of lentiviral vectors is similar in principle to derivation of oncoretroviral vectors.

The basic strategy is to separate the lentiviral vector from viral genes coding for the packaging proteins.

References:


Lentiviral Vector is Expressed in Lymphoid, Myeloid and CD34+ Progenies of SRC

pHR’GIN-MU3 Lentiviral Vector

Example of transgene expression in progenies of SRCs (candidate human hematopoietic stem cells) transduced with a lentiviral vector.

Successful transduction only required 2 days.

This demonstrated the most significant advantage of lentiviral vectors over oncoretroviral vectors. The risk of compromising stem cell characteristics is minimized during gene-modification of hematopoietic stem cells.

Reference:

Monitoring Expression of Transgenes/Reporter Genes

Reporter genes greatly facilitate monitoring of efficiency of transgene transduction and expression.
Until recently, the most popular reporter gene has been the bacterial neomycin phosphotransferase gene. It confers resistance to the neomycin analog, G418, in mammalian cells.

Genes coding for intracellular enzymes or cell surface proteins have also been used successfully.
About ten years ago, we developed a series of oncoretroviral vector backbones using antibiotic resistance genes as reporter genes.

Two standard ways to express the reporter gene:
1. Internal promoter (such as the mouse phosphoglycerate kinase promoter).
2. IRES (such as the encephalomyocarditis virus internal ribosome entry site).

Bob Hawley will elaborate on vector design and gene transfer methodologies during sessions of Lab 8.

Reference:

In recent years, fluorescent protein genes have become very popular reporter genes.

The first representative of fluorescent protein reporters was the green fluorescent protein (GFP) described in the bioluminescent jellyfish, Aequorea victoria. It is a protein of 238 amino acids.

Many spectral variants of GFP have been created, but none is a stable red fluorescent derivative.

Discovery of DsRed in 1999 expanded the range of colors toward the red end of the spectrum.

References:


Aequorea victoria is the bioluminescent jellyfish from which GFP was cloned.

Discosoma sp. is the non-bioluminescent reef coral from which DsRed was cloned.
GFP Chromophore

- GFP chromophore is intrinsic to the primary structure of the protein
- GFP fluorescence does not require substrates or cofactors
The GFP chromophore is located within an α-helix inside a compact β-barrel-like structure named a “beta-can”.

It has a cyclic tripeptide: Ser\textsuperscript{65}-Tyr\textsuperscript{66}-Gly\textsuperscript{67}.

**Reference:**

### Major Fluorescent Proteins and Mutagenized Variants

<table>
<thead>
<tr>
<th>Color</th>
<th>Protein</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Green</td>
<td>wtGFP</td>
<td>Original (wild type) GFP</td>
</tr>
<tr>
<td></td>
<td>EGFP</td>
<td>GFP variant with increased thermostability and fluorescence</td>
</tr>
<tr>
<td>Blue</td>
<td>ECFP</td>
<td>Cyan (blue-shifted) GFP spectral variant</td>
</tr>
<tr>
<td>Yellow</td>
<td>EYFP</td>
<td>Yellow/green (red-shifted) GFP spectral variant</td>
</tr>
<tr>
<td>Yellow</td>
<td>Venus</td>
<td>Matures faster and has increased fluorescence compared with EYFP</td>
</tr>
<tr>
<td>Yellow</td>
<td>Citrine</td>
<td>Improved photostability and sensitivity to pH changes compared with EYFP</td>
</tr>
<tr>
<td>Red</td>
<td>DsRed1</td>
<td>Red fluorescent protein</td>
</tr>
<tr>
<td>Red</td>
<td>DsRed2</td>
<td>Matures faster than DsRed1</td>
</tr>
<tr>
<td>Red</td>
<td>DsRed-T1/</td>
<td>Matures faster than previous DsRed variants</td>
</tr>
<tr>
<td></td>
<td>DsRed-Express</td>
<td></td>
</tr>
<tr>
<td>Red</td>
<td>mRFP1</td>
<td>Fast maturing, monomeric red fluorescent protein</td>
</tr>
<tr>
<td>Far red</td>
<td>HcRed</td>
<td>Far-red fluorescent protein</td>
</tr>
</tbody>
</table>


**wtGFP** is the original GFP.

**EGFP** is a synthetic, “humanized” derivative of one of the early GFP variants.

**ECFP** is a blue-shifted variant.

**EYFP** is a red-shifted variant.

**drFP583** (marketing name = DsRed) is the original red fluorescent protein.

**DsRedT1** (marketing name = DsRed-Express) is a much improved variant.

**HcRed** is a far-red fluorescent protein generated from a chromoprotein derived from the sea anemone *Heteractis crispa*.

**Reference:**

GFP and GFP-like Genes as Reporter Genes

- Non-invasive means to monitor expression
  - Particularly useful in primary cells and whole organisms
- Convenient detection and selection protocols
  - No need for substrates or antibodies
- Multiple spectral variants
  - Amenable to flow cytometric analysis of multiple genes
Examples of oncoretroviral vectors containing GFP variants.

**References:**


Examples of lentiviral vectors containing EGFP.

**Reference:**

Role of HOX11 in Leukemic Transformation

Translocations Involving the HOX11 and HOX11L2 Homeobox Genes Give Rise to T-Cell Acute Lymphoblastic Leukemia (T-ALL)


The next few slides show examples of applications in our group using fluorescent protein genes as reporter genes.

Observation: The homeobox gene HOX11 was found at the breakpoint of two chromosomal translocations in one type of T-ALL, implicating its role in the disease. The translocations juxtaposed TCR regulatory elements with HOX11, resulting in inappropriate HOX11 expression in T cells.

Objective: To confirm the role of HOX11 in leukemic transformation and understand the mechanism.

Reference:

HSC Immortalization and Leukemic Transformation

- Experimental design:
  - Expressed HOX11 in mouse hematopoietic stem cells using an oncoretroviral vector
  - Evaluated ectopic HOX11 expression in the *in vivo* mouse bone marrow transplantation model as well as an *in vitro* culture system

- Results:
  - T cell leukemia was induced *in vivo*, but only after a long latency (indicating that other genetic changes were involved)
  - Normal differentiation of myeloid progenitors was blocked *in vitro* (resulting in immortalization)

References:


Because HOX11 is involved in human T cell leukemia, we investigated the effect of dysregulated expression of HOX11 in mouse T cells, using the mouse fetal thymic organ culture model.

Experimental design: Expressed HOX11 (and EGFP) in mouse hematopoietic stem cells from day 14 fetal liver using an oncoretroviral vector, purified transduced cells by FACS, and evaluated ectopic HOX11 expression in mouse thymocytes.

Reference:

HOX11 is not expressed during normal T cell development.

In normal T cell development, hematopoietic stem cells migrate to the thymus and develop into thymocytes. Thymocytes progress through multiple DN (double-negative for CD4 and CD8) stages.

The four DN stages can be discriminated by the expression pattern of CD44 (receptor for hyaluronin) and CD25 (IL-2Rα).

Reference:

Results: Ectopic expression of HOX11 in mouse hematopoietic stem cells resulted in arrest of thymocyte development at DN1/DN2. However, unlike the effect of HOX11 in myeloid progenitors, this differentiation block did not result in immortalization.

In order to identify downstream targets of HOX11, we turned to human T cell lines.

A useful strategy utilizes regulatable expression of HOX11.
Regulatable gene expression systems:
1. Bacterial Cre-lox recombination system – irreversible regulation.
2. Tetracycline-inducible expression system – reversible regulation.

References:


Inducible Expression of HOX11 in a Human T Cell Line

Example of inducible expression of HOX11 in a human T cell line transduced with lentiviral vectors containing the ECFP and EGFP reporter genes. Expression of ECFP correlated with a regulatory transcription factor (rtTA). Expression of EGFP correlated with HOX11.

Microarray studies can be conducted using RNAs isolated from HOX11-induced and uninduced cells.
Co-operative Effects of Multiple Genes in Leukemic Transformation of Human Hematopoietic Progenitor Cells

- Disrupt differentiation
- Regulate cell cycle progression
- Prevent apoptosis
- Preserve the length of telomeres

Our earlier studies also suggested that other genetic changes co-operated with HOX11 in leukemic transformation.

*In vitro* model system: Transduce human hematopoietic progenitor cells with multiple genes in an attempt to recapitulate the process of leukemic transformation.

Linking each transgene to a fluorescent protein reporter gene greatly facilitates the monitoring of transgene expression.

**Reference:**

An on-going experiment in our group showing expression of three fluorescent protein reporter genes, indicating expression of three transgenes.

Row 1 = cells expressing EYFP
Row 2 = cells expressing EGFP/EYFP
Row 3 = cells expressing EGFP/EYFP/DsRed

ECFP will be used with a fourth transgene.
Summary

• Transfer genes to hematopoietic stem and progenitor cells using oncoretroviral and lentiviral vectors

• Monitor gene transfer and expression using fluorescent protein genes as reporter genes
Preview of Lab 8

Multiple Gene Expression
Each of these oncoretroviral vectors carries a fluorescent protein gene and the neomycin phosphotransferase gene conferring resistance to the neomycin analog, G418.

To obtain cells expressing the individual fluorescent protein genes, either FACS or selection in G418 is adequate.

To obtain cells expressing multiple fluorescent protein genes, FACS is necessary (since all vectors contain the neomycin phosphotransferase gene).

**Reference:**

Derivation of Cells Stably Expressing Individual or Multiple Fluorescent Protein Genes

• Packaging cell line: GP+E-86 containing gag, pol and env
• Transfect GP+E-86 with oncoretroviral vectors containing ECFP, EGFP, EYFP or DsRed cDNAs
• Select in G418 (Geneticin) to derive stable cell lines expressing the individual fluorescent protein genes: 0, C, G, Y, R

• Target cell line: Sp2/0-Ag14
• Transduce Sp2/0-Ag14 with supernatant containing different combinations of fluorescent protein retroviral vector particles
• Select in G418 and/or by FACS to derive stable cell lines expressing individual or multiple fluorescent protein genes: 0, C, G, Y, R, CY, GY, CGY, GYR, CGYR

GP+E-86 is a mouse fibroblast cell line.

Sp2/0-Ag14 is a mouse hybridoma cell line.

References:


Fluorescence microscopic images of GP+E-86 cells expressing the individual fluorescent protein genes.

Reference:

A useful tool in assessing spillover estimates is the BD Fluorescence Spectrum Viewer developed by Dr. Joe Trotter (BD Biosciences).

Go to http://wwwbdbiosciences.com/spectra.
Simultaneous analysis of ECFP, EGFP, EYFP and DsRed using dual excitation wavelengths of 458 nm and 568 nm.

Reference:


Simultaneous analysis of ECFP, EGFP, EYFP and DsRed using dual excitation wavelengths of 488 nm and 407 nm.

Reference:


3D views (WinList software, Verity Software House) of a mixture of ten populations of Sp2/0-Ag14 cells expressing the individual or multiple fluorescent proteins genes.

The ten populations consist of cells expressing:
1. No fluorescent protein: gray
2. ECFP: purple
3. EGFP: green
4. EYFP: yellow
5. DsRed: red
6. ECFP/EYFP: light blue
7. EGFP/EYFP: turquoise
8. ECFP/EGFP/EYFP: orange
9. EGFP/EYFP/DsRed: brown
10. ECFP/EGFP/EYFP/DsRed: dark blue

Top left view: CFP vs. GFP vs. YFP
Top right view: GFP vs. YFP vs. DsRed
Bottom left view: CFP vs. GFP vs. DsRed
Bottom right view: CFP vs. YFP vs. DsRed
Log transform is the traditional format for displaying data on a logarithmic scale.

Biexponential transform is a new display developed by Dr. Dave Parks and Wayne Moore at Stanford University.

HyperLog transform is a new display developed by Dr. C. Bruce Bagwell at Verity Software House.
Examples of log transform (FACSDiva software, BD Biosciences).

0 = Sp2/0 cells not expressing any fluorescent protein
C = cells expressing ECFP
G = cells expressing EGFP
Y = cells expressing EYFP
R = cells expressing DsRed
CY = cells expressing ECFP/EYFP
GY = cells expressing EGFP/EYFP
CGY = cells expressing ECFP/EGFP/EYFP
GYR = cells expressing EGFP/EYFP/DsRed
CGYR = cells expressing ECFP/EGFP/EYFP/DsRed

Rows 1 & 2 = mixture of 0, C, G, Y and R, without or with compensation for spectral overlap, respectively
Rows 3 & 4 = mixture of 0, C, G, Y, R and CGYR, without or with compensation for spectral overlap, respectively
Rows 5 & 6 = mixture of 0, C, G, Y, R, CY, GY, CGY, GYR and CGYR, without or with compensation for spectral overlap, respectively
Examples of biexponential transform* (FACSDiva software).

- 0 = Sp2/0 cells not expressing any fluorescent protein
- C = cells expressing ECFP
- G = cells expressing EGFP
- Y = cells expressing EYFP
- R = cells expressing DsRed
- CY = cells expressing ECFP/EYFP
- GY = cells expressing EGFP/EYFP
- CGY = cells expressing ECFP/EGFP/EYFP
- GYR = cells expressing EGFP/EYFP/DsRed
- CGYR = cells expressing ECFP/EGFP/EYFP/DsRed

Rows 1 & 2 = mixture of 0, C, G, Y and R, without or with compensation for spectral overlap, respectively
Rows 3 & 4 = mixture of 0, C, G, Y, R and CGYR, without or with compensation for spectral overlap, respectively
Rows 5 & 6 = mixture of 0, C, G, Y, R, CY, GY, CGY, GYR and CGYR, without or with compensation for spectral overlap, respectively

*The software used for this experiment is a beta version of the BD FACSDiva software that is not yet commercially released.
Examples of HyperLog transform (WinList software).

0 = Sp2/0 cells not expressing any fluorescent protein
C = cells expressing ECFP
G = cells expressing EGFP
Y = cells expressing EYFP
R = cells expressing DsRed
CY = cells expressing ECFP/EYFP
GY = cells expressing EGFP/EYFP
CGY = cells expressing ECFP/EGFP/EYFP
GYR = cells expressing EGFP/EYFP/DsRed
CGYR = cells expressing ECFP/EGFP/EYFP/DsRed

Rows 1 & 2 = mixture of 0, C, G, Y and R, without or with compensation for spectral overlap, respectively
Rows 3 & 4 = mixture of 0, C, G, Y, R and CGYR, without or with compensation for spectral overlap, respectively
Rows 5 & 6 = mixture of 0, C, G, Y, R, CY, GY, CGY, GYR and CGYR, without or with compensation for spectral overlap, respectively
Examples of FCOM histograms (WinList software).

0 = Sp2/0 cells not expressing any fluorescent protein  
C = cells expressing ECFP  
G = cells expressing EGFP  
Y = cells expressing EYFP  
R = cells expressing DsRed  
CY = cells expressing ECFP/EYFP  
GY = cells expressing EGFP/EYFP  
CGY = cells expressing ECFP/EGFP/EYFP  
GYR = cells expressing EGFP/EYFP/DsRed  
CGYR = cells expressing ECFP/EGFP/EYFP/DsRed

Row 1 = mixture of 0, C, G, Y and R  
Row 2 = mixture of 0, C, G, Y, R and CGYR  
Row 3 = mixture of 0, C, G, Y, R, CY, GY, CGY, GYR and CGYR
Acknowledgments

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Medical Center
Review Articles


Selected References: Monitoring Gene Transfer and Expression

Monitoring Gene Transfer and Expression


Selected References: Fluorescent Proteins

Fluorescent Proteins

Flow Cytometric Strategies for Detection of Fluorescent Proteins


Selected References: Fluorescence Compensation and Data Display

Fluorescence Compensation and Data Display


