Molecular Biology: methods and applications

On reserve reference: An Introduction to Genetic Analysis
11th Ed. Griffiths et al.

Fall 2019
CORE

Deborah Otteson, Ph.D.
UH College of Optometry
I do not have any conflicts or financial interest in any companies or products identified in these lectures. The information provided is for educational purposes for this class.
Transcriptome: The collection of all RNA transcripts in a cell.

Proteome: Collection of all proteins in a cell or tissue.

The genome is identical in all cells.

Griffiths 10th edition
Figs. 1-2 & 1-5
“Central Dogma” of Gene Expression and Protein Synthesis

RNA viruses: Reverse transcription (e.g. HIV, retroviruses)

(a) Replication (DNA synthesis)
Transcription (DNA synthesis) → mRNA → Translation (protein synthesis)

(b) DNA → Transcription (RNA synthesis) → mRNA → Translation (protein synthesis)
Applications of Molecular Biology and Genetics in Medicine

• Understand processes regulate normal and abnormal function in cells and tissues

• Identify and understand genetic factors that cause disease

• Predict susceptible individuals/prevention

• Develop gene replacement/corrective therapies

• Identify new drug/treatment targets

• Identify patients who will benefit from different therapies (different causes = different treatments)
What is a gene?

• **Gene: Functional Definition**
  - A unit of heredity which is transferred from a parent to offspring and determines some characteristic of the offspring.

• **Gene: Molecular Definition**
  - A distinct sequence of nucleotides within a chromosome, the order of which determines the order of monomers in a polypeptide or nucleic acid molecule which an organism may synthesize.

• **Chromosomes contain the genes**
  - Humans: 20-25K protein coding genes in genome

**Figure 1-13b**
Introduction to Genetic Analysis, Eleventh Edition

Arrows: Fluorescently labeled genes in condensed human chromosomes
• Contributions from genotype/environment varies with disease
• Some diseases have both genetic and environmental components
  e.g. macular degeneration; cataract; heart disease
• Mutations in a single gene may result in different clinical presentations
• The same clinical presentation or ‘disease’ may result from mutations in multiple, different genes
Retinal Disease Associated Genes

So-called “disease genes” have normal functions in cells
It is the MUTATION or MALFUNCTION of the genes that can cause disease
Press Release

FDA Approves Spark Therapeutics’ LUXTURNA™ (voretigene neparvovec-rzyl), a One-time Gene Therapy for Patients with Confirmed Biallelic RPE65 Mutation-associated Retinal Dystrophy

LUXTURNA is first gene therapy for a genetic disease, first and only pharmacologic treatment for an inherited retinal disease (IRD) and first adeno-associated virus (AAV) vector gene therapy approved in U.S.

https://focus.masseyeandear.org/making-gene-therapy-history/

Links to other videos:
https://www.biointeractive.org/classroom-resources/genes-medicine
https://focus.masseyeandear.org/two-months-after-gene-therapy-jack-sees-a-little-brighter-more-clearly/
Leber Congenital Amaurosis (LCA)

- Most common cause of inherited blindness in childhood (2-3 out of every 100,000 babies)

- Causes severe vision loss at birth resulting from dysfunction of rods and cones

- May include other vision problems, including photophobia, nystagmus, and extreme hyperopia, keratoconus

- Mutations in at least 14 genes can result in LCA; cause unknown in 30% LCA

- Inherited in an autosomal recessive manner
  - Both parents must carry a defective gene for the condition in order to pass it on to their children.
  - Each of their children has a 25 percent chance of inheriting the two LCA genes (one from each parent) needed to cause the disorder
Mutations in Genes Regulating Retinoid Cycle Result in Retinal Degenerative Diseases

LCA = Leber congenital amaurosis
RP = Retinitis pigmentosa
AMD = Age-related Macular Degeneration
CSNB = congenital stationary night blindness
Stargardt’s
Fundus albipunctatus

Mutational Analysis of Patients with Autosomal Recessive Childhood-onset Leber Congenital Amaurosis (LCA)

Diagnosis: LCA

Strategies: Analysis of genomic DNA from patients and unaffected individuals

1. Linkage study of families
2. GWAS
3. Candidate gene/mutation identification
Definitions

• **Linkage:** Traits (SNPs genes, phenotypes) that are inherited together more frequently than predicted by chance alone.
  – Two traits (e.g., disease and SNP) that are physically close within a chromosome are more likely to be inherited together. So they are ‘linked’ (remember independent assortment of chromosomes?)

• **SNP:** single nucleotide polymorphism.
  – Single nucleotides within the genome that vary between individuals
  – Often found outside of genes
  – Used in mapping gene/disease locations
  – May have no health effects themselves, but are located near a potential genetic factor involved in disease
Family Linkage Studies: Gene Discovery for Inherited Diseases

Identify family pedigrees and determine patterns of inheritance for disease.

Identify genetic markers (SNPs) in patient and family members that are inherited with same pattern as disease trait

Requires extended families, defined diagnostic criteria

Tan et al; 2014
Genome-wide association studies in neurology
GWAS: Gene Discovery for Rare or Multifactorial Diseases

What to do if you can’t find enough large families for pedigree and family linkage studies?

**Genome wide association studies (GWAS)** are used to look for disease associated changes in large populations of unrelated affected and unaffected individuals.
Genetic Mapping and Mutation Analysis Requires Genomic DNA from Affected and Unaffected Subjects

Genomic DNA needed for genetic analysis, identification of mutation and variations

- Isolate DNA from patient/subject
  - Blood
  - Cheek swab
  - Biopsy (e.g. cancer)
  - Saliva

- DNA in all cells should be the same

http://www.alleight.com/Products/Epicentre/BuccalAmp.htm
Double-stranded alpha helix

- Deoxyribose backbone (blue)
- Base pairs inside helix

2 types of bases (purines and pyrimidines)

Invariant pairing: AT  GC

So, if you know the sequence of bases on one strand, you can predict the sequence on the opposite strand.
Watson-Crick Pairing of Bases in DNA

Invariate pairing bases

- Each base pair consists of
- 1 purine and 1 pyrimidine

\[
\begin{align*}
\text{T} - \text{A} \quad &\text{(DNA)} \\
\text{U} - \text{A} \quad &\text{(RNA)} \\
&\quad = 2 \text{ hydrogen bonds}
\end{align*}
\]

\[
\begin{align*}
\text{C} - \text{G} \quad &\text{(DNA & RNA)} \\
&\quad 3 \text{ hydrogen bonds}
\end{align*}
\]
Microarrays

- Array contains tens of thousands of spots of single stranded DNA oligonucleotides
- Many copies of one specific sequence variant per spot
- All possible sequence variants of SNPs are included as separate spots

Expected sequence:

- **A T G C A G C C T C T G T C A G**
- **A T G C A G C C T C T G T C A G**

Variant (SNP, mutation)

- **A T G C A G C C T T G T C A G**

URLs:
- [www.stemcore.ca/services/microarrays](http://www.stemcore.ca/services/microarrays)
- [www.ur.umich.edu/0506/Oct17_05/02.shtml](http://www.ur.umich.edu/0506/Oct17_05/02.shtml)

Microarrays

1. Patient DNA is isolated, fragmented, and labeled
2. Genomic DNA denatured to make sample single stranded
3. Apply to microarray; allow hybridization (formation double stranded DNA)
4. Perfect match will hybridize, imperfect match washes off
5. Detect fluorescence of probe hybridized to specific spots

Expected sequence: $\text{ATGCAGCCTCTGTCAG}$

Variant (SNP, mutation): $\text{ATGCAGCCTTTGTCAG}$

http://www.stemcore.ca/services/microarrays
http://www.ur.umich.edu/0506/Oct17_05/02.shtml

https://en.wikipedia.org/wiki/DNA_microarray
Microarrays

1. **For GWAS/genotyping:** contain all possible sequence variants of known SNPs (Test sample: labeled genomic DNA from subjects)

2. **For mutation analysis and genotyping:** contain all known or potential sequence or splice variants in disease associated genes (Test sample: labeled genomic DNA from subjects)

3. **For gene expression analysis:** contains DNA copies (cDNA) for mRNAs or DNA copies of exons of known or predicted genes (Test sample: labeled cDNA from subjects)

Expected sequence:

- **Expected sequence:**
  - A T G C A G C C T C T G T C A G
  - A T G C A G C C T C T G T C A G

*Variant (SNP, mutation)*

- A T G C A G C C T T G T C A G

.http://www.stemcore.ca/services/microarrays

https://en.wikipedia.org/wiki/DNA_microarray
Linkage analysis of affected and unaffected family members

SNPs present across all chromosomes
Location and order known

Determine alleles present in each

Diploid = 2 sets per subject

Gray box shows adjacent clusters of SNP alleles that co-segregate with disease

Map back to genome

Does not identify specific gene, only the region containing the gene contributing to the trait/disease
GWAS

Looks for SNPs that are more frequently present in patients with disease than in normal controls.

Knowing location of the SNP in the genome, we can place the disease trait nearby.

Does not identify specific gene, only the region containing the gene contributing to the trait/disease.

https://www.genome.gov/20019523/genomewide-association-studies-fact-sheet/
“Manhattan plots” correlate genes to a trait or illness

Source: Nature Genetics

- Compile all SNP variants across all subjects
- Identify over-represented variants in affected population (arrows)
- Identify genes associated with SNP
- Look for mutations in candidate genes

Frequency of SNP variants in individuals with trait under investigation

Mutations in RPE65 cause autosomal recessive childhood-onset severe retinal dystrophy

Su-min Gu, Debra A. Thompson, C.R. Srisailapathy Srikumari, Birgit Lorenz, Ulrich Finckh, Aileen Nicoletti, K.R. Murthy, Michaela Rathmann, Govindasamy Kumaramanickavel, Michael J. Denton & Andreas Gal

Nature Genetics 17, 194–197 (1997) | Download Citation

- Results of family linkage analysis of patients
- Linked LCA to SNPs on Chromosome 1
- Region contains RPE65 gene
- NEXT: Identify specific mutations that fit the pattern of inheritance in families with disease and would be predicted to alter the protein
Gene organization and mRNA splicing

Splicing removes introns

Mature mRNA (contains copy of exons)
Looking for mutations in *RPE65*

1. Design exon specific primers FOR EVERY EXON of gene
   - Remember: Exons contain the protein coding portion of the gene. So, linkage analysis looks for changes in exons

2. **PCR amplify** genomic DNA between primers (include affected vs unaffected)

3. **Sequence** PCR products

4. Look for sequence variants (mutations)
   - Are variants always present with disease?
   - Are variants predicted to alter protein sequence and cause problems with protein structure and/or function?
PCR: Polymerase Chain Reaction

Taq DNA polymerase
Isolated from *Thermus aquaticus* (bacterial)
Heat stable
Some Taq enzymes also have proofreading capability

https://www.biointeractive.org/classroom-resources/polymerase-chain-reaction-pcr
**PCR: Polymerase Chain Reaction**

**Key components of PCR reaction:**
- Template DNA (cDNA, genomic DNA)
- Target-specific primers
- Deoxyribonucleotides
- Taq DNA polymerase

**Primers are designed to confer specificity: only desired target is amplified**

**Figure 10-3 part 1**

**Figure 10-3 part 2**
**RPE65 genotyping: DNA Sequencing of PCR Products**

**Chain Termination Method (Sanger Method)**

**Di-deoxy sequencing method**

Di-deoxyNTP (ddATP; ddCTP ddGTP ddTTP)
- Lacks -OH group on 3' carbon
- Cannot form a phosphodiester bond with the next nucleotide in the growing chain
- DNA synthesis is terminated

Video of sequencing:
https://www.youtube.com/watch?v=jFCD8Q6qSTM
• 1 reaction for each ddNTP (ddATP, ddTTP, ddCTP, ddGTP)
• Generates all possible fragments with each labeled at one end
• Shown: DNA Fragments generated by di-deoxy sequencing using ddATP with fluorescent tag (★)
Polyacrylamide Gel Electrophoresis (PAGE) for DNA Sequencing

Separate reaction products from each ddNTP reaction (4 reactions needed)

Use polyacrylamide gel electrophoresis (PAGE) to separate fragments by size

- Gel provides porous matrix (functions like sieve)
  - Small molecules move quickly: migrate to bottom of gel
  - Large molecules move slowly: migrate higher in gel
- Add reaction mixes into adjacent wells at negative pole
- Electrical current flows thru buffers and gel
- Single base resolution of fragment size
- Read sequence from bottom (small) to top (large) across 4 ddNTP reactions
Automated Chain Termination DNA Sequencing

- Automated sequencer uses single reactions with 4 labeled ddNTPs
  - Each ddNTP has different fluorescent label
- Electrophoresis of products uses a gel inside a thin capillary tube
- Reads fluorescent signal as each product passes detector
- Electropherogram (below) shows fluorescence intensity for each PCR product
  - Each peak shows a fragment with a specific ddNTP at the end
  - Read sequence from left to right (small to large fragments)
QUESTION: What proteins in RPE are involved in retinoid recycling?

1. Isolate protein from bovine retinal pigmented epithelium (RPE) cells
   - Why use bovine RPE?
2. Generated many antibodies against RPE proteins
3. Test antibodies in Western blot and identify proteins recognized
4. Isolate protein of interest and determine sequence
5. Back translate to predict RNA and DNA sequence of gene
6. Use predicted sequence to clone gene
Protein Structure: Basics

- Proteins are polypeptide polymers of amino acids
  - Side chains of different amino acids have different chemical characteristics

- Synthesized in cell by ribosomes that translate mRNA into protein
  - Free ribosomes make cytoplasmic proteins
  - Ribosomes on rough endoplasmic reticulum (ER) make membrane bound and secreted proteins
Translation Basics: Genetic Code

- Amino acids are encoded by base triplets called codons
- Many amino acids are encoded by more than one codon (degenerate)
- Sequence of DNA or RNA can be used to predict protein sequence based on genetic code
Protein Basics: Primary Structure

Each amino acid called “residue” (R)

Each AA is numbered: starting at #1 at N terminal (amino end), with sequential numbering to C-terminal end.

Co-linearity of DNA, RNA and protein sequence

Amino acids linked by peptide bonds

Multiple amino acids linked together form a polypeptide

Properties of amino acid side chains confer structure and function of protein
Protein Basics: Protein folding

**Secondary structure**
Polypeptides can bend into regularly repeating structures created by *hydrogen bonds* between different amino acid residues

**Tertiary structure** = 3-dimensional architecture
Chaperon proteins in ER can regulate protein folding. Stabilized by various intermolecular bonds
Proteins that are incorrectly folded are refolded or degraded

**Quaternary structure = Multimeric Proteins**
Many mature proteins are composed of multiple subunits
Joined by various intramolecular bonds
Subunits: separate proteins, may be encoded by the same or different genes

<table>
<thead>
<tr>
<th># subunits</th>
<th>Dimer</th>
<th>Trimer</th>
<th>Tetramer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
</tbody>
</table>

Homodimer = 2 identical subunits
Heterodimer = 2 different subunits
Western Blot Analysis of Proteins: Polyacrylamide gel electrophoresis (PAGE)

1. Isolate proteins from sample
2. Denature proteins: eliminate secondary, tertiary, quaternary structure
   - Break chemical bonds by heating
   - Prevent refolding by neutralizing charges with SDS (detergent)
3. Separate proteins by polyacrylamide gel electrophoresis (PAGE)
   - Gel provides porous matrix (functions like sieve)
   - Add proteins at negative pole
   - Electrical current flows thru buffers and gel
   - Small proteins move easily, large proteins move slowly
   - Include known size standards for comparison
Western Blot Analysis of Proteins: Transfer proteins to filter

4. Blot = transfer to filter
   - Make ‘sandwich’ of gel and specialized membrane filter
   - Place into buffer
   - Electrical current moves proteins from gel to membrane
   - Proteins ‘stick’ to membrane, but do not pass thru
Western Blot Analysis of Proteins: Detect protein of interest with antibodies

5. Remove gel from membrane

6. Process with various solutions to:
   - Block non-specific protein binding
   - Apply Primary Antibody
     • Binds to protein of interest
   - Wash
   - Apply secondary antibody
     • Used to detect primary antibody
     • Has chemical tag that allows detection

7. Analyze band for protein of interest
   - Determine size by comparison to known standards run on original gel
Protein Analysis of RPE65
PAGE and Western Blot

A: Polyacrylamide gel
- Stained to show proteins
- Reference ‘ladders’ of known size proteins

B: Western blot
- Proteins from bovine RPE (total protein and membrane protein fraction)
- PAGE and blot
- Probed with antibody against unknown RPE protein
- Antibody detects 65 kDa protein
Some Applications of Western Blots

• Medical diagnosis (some examples listed)
  – HIV infection,
  – Hepatitis B Virus infections
  – Bovine Spongiform Encephalopathy (“mad cow disease”)
  – Lyme disease
• Detect presence of protein in a mixture of other proteins
• Determine size and test for post-translational modifications of a protein
• Analysis of protein-protein interactions
• Determine protein stability
• Structure domain analysis
Functional analysis: *RPE65*

- RPE65 identified as an abundant protein in RPE
  - Recognized as 65 kDa protein by the one of the antibodies using **WESTERN BLOTS**
  - Initially function was unknown
  - Predicted to be involved in retinoid processing by similarity to known enzymes

- Clone gene to study RPE65 function
  - Sequence protein
  - Determine corresponding RNA/DNA sequences
  - Clone *RPE65* gene
Cloning RPE65: Why Clone Genes?

• Use as probes for screening libraries, Southerns, northerns
• Determine gene structure
• Functional analysis
  • overexpress gene in cells
  • generate large amounts of protein for biochemical studies
• Mutational analysis (mutate gene, test function)
• Generate transgenic animals for *in vivo* studies
• Develop gene replacement therapies
Cloning RPE65 Gene from RPE mRNA

Method 1: clone from cDNA library

• Useful if you don’t know full gene sequence
• Less error prone (fewer mutations generated during preparation)
• Slow (multi-step)

Method 2: clone by RT-PCR (reverse transcriptase PCR)

• Most efficient if you know sequence and gene structure
• Repeated PCR can cause mutations
• Faster
• May eventually have to clone cDNA into plasmids/virus, depending on application
Gene organization and mRNA splicing

- Splicing removes introns

Mature mRNA (contains only exons)

- mRNA contains protein coding regions
- RNA is unstable and easily degraded
- For cloning, need to make DNA copy of RNA
- Method: Reverse transcription
RNA vs DNA Basics

RNA
Single stranded

5' → 3'

A, C, G, U

Ribose sugar

RNA

DNA
Double stranded
Anti-parallel strands

5' → 3'

3' ← 5'

Sense strand/non-template
Anti-sense strand/template

A, C, G, T

Deoxyribose sugar

DNA

Single stranded nucleic acids want to hybridize (form duplexes) with reverse complementary nucleic acids: DNA/DNA or DNA/RNA or RNA/RNA
Analysis of RNA: Making cDNA libraries

1. Isolate RNA
2. Reverse transcription (RT)
   - Uses DNA primers to bind RNA
   - Viral reverse transcriptase enzyme makes DNA copy of RNA = cDNA
     • DNA synthesis is 5’ to 3’, starting at primer
   - RNAse removes RNA strand from DNA/RNA duplex
   - DNA polymerase synthesizes second strand of DNA (double stranded cDNA)

Primers used for RT:
Oligo dT (5’-TTTTTTTTT-3’)
• Bind to polyA tail of mRNA
• Allows RT of mRNA only
• Used to generate full length cDNAs, starting at 3’ end of transcript

Random primers (5’-NNNNNN-3’)
• Bind to any RNA at any complementary sequence
• Allows RT of all RNAs
• Generates shorter cDNAs (not full length)
3. Cut cDNA and vector (plasmid) with restriction enzymes and clone into plasmid or virus vector.
Basic Molecular Methods: Restriction Enzymes

Bacterial enzymes

Normal function: defend against phage infection
Recognizes and cuts (digests) specific DNA sequences

e.g., EcoRI (from E. coli)

\[
\begin{align*}
5'\text{GAATTC} & \quad 3' \\
3'\text{CTTAAG} & \quad 5'
\end{align*}
\]

Recognition sequences methylated in the bacterial genome to prevent DNA digestion by restriction enzymes made by the cell
Wide range of enzymes with different target recognition sites
Commercially available
Cloning Using Restriction Enzymes

Cut plasmid and cDNA insert(s) with same restriction enzyme
- Plasmid is circular DNA that can replicate in bacteria
- Carry antibiotic resistance genes in bacteria

Restriction digest generates compatible “sticky ends” on ends of plasmid and cDNA insert(s)

Overhangs find match and anneal (hybridize)

Bacterial enzyme repairs nicked ends

Grow plasmid in *E. coli*
4. Transform and plate plasmids with cDNA inserts

- Add to bacterial cells (usually *E. coli*)

- Grow bacteria containing plasmids

- Growth conditions are optimized so only bacteria containing plasmid will grow
  - Antibiotics in media prevent growth of *E. coli* that do not have plasmid
Screening cDNA library (modified Southern blot): Identifying desired clone

1. **Plate library (plasmids in E coli)**
   - Use filters to take ‘lifts’ of colonies
   - Filters processed to remove proteins and expose DNA from plasmids

What is a Southern blot???

General Methods DNA, mRNA and Protein Analysis

Gene (DNA)-Southern blot
Transcription (RNA)-northern blot
Translation (protein)-western blot

General strategy:
• Isolate appropriate cellular component
• Size fractionate by gel electrophoresis
  – Can use PAGE or agarose gels
  – Electrical current drives molecules through gel
  – Small molecules migrate fast, large molecules migrate slowly
• Transfer to filter
• Detect target of interest using labeled probes
• Analyze: compare wild-type vs. experimental, disease vs. healthy, etc
Some Applications of Southern Blots (DNA)

- Identification of a single gene in a pool of DNA fragments
- Detection of specific DNA sequences in a genome
- Studying genomic deletions, duplications, and mutations that cause various diseases
- Genotyping (e.g. transgenic mice)
- Detection of genetic diseases and cancers, such as monoclonal leukemia and sickle cell mutations
- DNA fingerprinting and forensic tests such as paternity testing and sex determination

Inventor: Dr. E. Southern (published 1975). Northern, western named to go with Southern name for DNA method.
Some Applications of Northern Blots (RNA)

• Determine actual size of transcript(s) and reveal presence of alternative splicing in a single gene

• Gene expression studies – e.g. to observe overexpression of cancer-causing genes and gene expression in case of transplant rejection

• In diagnosis of several diseases, e.g. Crohn’s disease

• For detection of viral microRNAs that play key roles in viral infection

• To screen recombinants - by detecting the mRNA formed by the transgene

• Requires that you have RNA from the tissue where it is expressed!!!!

Reverse-transcriptase PCR (RT-PCR) can provide a faster and more sensitive alternative
Create Probes for Southern blot analysis: Identify cDNA clone containing **RPE65**

2. Make probe and hybridize to filters
   - For cloning RPE65, researchers used the protein sequence to predict DNA sequence (back translation using codon table!)
   - Chemically synthesize **DNA oligonucleotide probes** for all possible back translated sequences
   - **Label probe** with enzyme or radioactivity for detection
   - **Denature DNA** on filters and probe to make single stranded
   - **Hybridize**: probe anneals to target only
   - **Wash** off unbound probe from filter

Translation Basics: Genetic Code

- Back translation from protein to RNA or DNA gives multiple possible RNA/DNA sequences

Example: Protein = Met-Pro-Trp

RNA: AUGCCGUUGG  DNA: ATGCCCCTTGG
AUGCCCGUUGG  ATGCCCCTTGG
AUGCCCAUGG  ATGCCCATTGG
AUGCCCGUGGG  ATGCCCTTGG

- For screening, need to generate multiple DNA probes using all possible sequences
Screening cDNA library (modified Southern blot)

3. **Identify colonies containing plasmid with desired sequence**

- For radioactive probes: expose filters to film
- For chemically labeled probes: process filter with appropriate reagents
  - For both, hybridization of probe to target clone will show as spot

- Match spot on film or filter to plate and pick corresponding colony
- Grow bacteria and isolate plasmid DNA
- Sequence to verify gene is correct one

Method 2: Clone Genes by RT – PCR

Uses available sequence information for gene of interest:
- Design PCR primers coding region of transcript
- Primers must be located in 5’ and 3’ UTR to clone full coding region

1. Isolate RNA from tissue of interest
2. Use reverse transcriptase to generate cDNA
3. PCR amplify using gene-specific primers
   - Uses ‘proof-reading’ taq polymerase to reduce introduction of mutations in PCR products
4. Run PCR product on gel
5. Purify PCR product and sequence verify
6. Depending on final use, PCR product can be cloned into various vectors
Comparison of Ocular Pathologies in Vitamin A–Deficient Mice and RPE65 Gene Knockout Mice

Yang Hu, Ying Chen, Gennadiy Moiseyev, Yusuke Takahashi, Robert Mott, and Jian-xing Ma

Investigative Ophthalmology & Visual Science, July 2011, Vol. 52, No. 8
Copyright 2011 The Association for Research in Vision and Ophthalmology, Inc.

Entrainment of circadian rhythm to a photoperiod reversal shows retinal dystrophy in RPE65−/− mice

D.M.Daniels a,b *, C.W.Stoddart a,b, M.T.Martin-Iverson b,c, C.-M.Lai d, T.M.Redmond d, P.E.Rakoczy e


Cone opsin mislocalization in Rpe65−/− mice: a defect that can be corrected by 11-cis retinal.

Rohrer B a, Lohr HR, Humphries P, Redmond TM, Seeliger MW, Crouch RK.
Developing Animal Model(s)

Why make Genetically Modified Organisms (GMO)

Develop research tools
   Develop models for studying disease or gene function
   Replace normal genes with mutated genes to determine consequences of specific mutations
      e.g. RPE 65 knockouts: study LCA
      MMP-9 knockouts to study dry eye disease
      Mutant rhodopsin knock-ins to model Retinitis pigmentosa

Also used to produce therapeutic proteins, to modify plants/animals for production
   Transgenic sheep and goats to produce human proteins in their milk
   Insulin production
   Herbicide resistant plants
Transgenic Technology

Transgenic

general term for genetically modified animal;
typically refers to random gene insertion

Knock-out

transgenic animal with a specific gene deleted/alktered in the genome

Knock-in

transgenic animal designed to replace a target gene and with foreign gene

Conditional Knockout

transgenic animal that is engineered to delete/alter a target gene only under certain conditions

John Wilson
http://www.bcm.edu/biochem/?PMID=3946


Transgenics: Overexpression of Genes

Advantages:
• Fast, can generate many animals (weeks)
• Normal gene still intact
• Can introduce foreign genes
• Promoters can confer specificity
• Variable number of repeats can enable ‘dose’ studies

Disadvantages:
• Insertion location random
• Normal gene still intact
• May disrupt unknown genes (off target effects)
• Need to analyze multiple offspring since insertion site may alter expression of transgene
Transgenics: Overexpression of Genes

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Generating Targeted Gene Modification

Replace original gene with engineered gene of choice

- Uses cellular DNA repair mechanisms to replace endogenous gene with the engineered targeting construct
- Homologous recombination is a rare event
- Much more difficult, time consuming
- Can take up to a year or more until you have mice to analyse
- For some genes, deletion can be lethal (hard to study vision if mouse dies too soon).
- Various methods used to make conditional knockouts to limit the cells and timing of gene deletion/alteration
- Newly developed methods (CRISPR) can be used to directly edit gene in chromosome. (not discussed in this course)
Generating Targeted Gene Modification

1. Embryonic Stem cells (ES cells) from blasocyst of brown mouse

2. Clone targeting construct
   - Generate construct containing mouse genomic DNA that surrounds targeted region
   - Contains DNA is engineered to include desired mutation/insert/deletion
   - Can add other genes to replace coding region of gene (these will be expressed under control of gene promoter)
   - Additional genes are added for drug selection of cells with homologous recombination
3. Introduce targeting construct into ES cells = **transfection of plasmid DNA** (also can use **infection** to transfer if DNA is cloned into **virus**)

Start with $10^8$ embryonic stem cells

- 5-30 % of cells don’t take up any DNA
- In cells that take up the DNA the gene is not replaced:
  - Most frequent: transgene DNA not incorporated into genome
  - Others: transgene is randomly insertions

4. **Homologous recombination**: ~1 cell in 100 million will have homologous recombination event (so, for 1 experiment you may only recover ~10 cells!)
5. Drug selection of ES cells with homologous recombination: Only cells with new DNA inserted correctly at target location will survive drug treatment.
Generating Mice with Targeted Gene Modification

6. **Inject ES cells** with targeted gene (from brown mouse) **into blastocysts** from white mouse (contains mixture of normal and targeted cells)

7. **Transplant blastocysts** into female host

8. **Pups will be chimeras** (brown and white)

http://sgugenetics.pbworks.com/w/page/24704977/Creation%20of%20a%20%22Knockout%20Mouse%22
Chimeras

- Chimeras contain mixture of cells with and without transgene
- Somatic cells containing transgene: not passed to next generation
- Germline cells containing transgene: passed to next generation

Mythical Chimera

Chimeric mouse

http://intramural.nimh.nih.gov/tgc/photogallery.html

chimera.clubhertzog.com/images/chimera.png
Generating Mice with Targeted Gene Modification

Breed chimeras to normal mice

8. **Genotype pups** to identify mice with targeted gene

9. **Breed** to generate homozygous mice

10. **Analyze phenotypes:**
    Determine effects of gene knockout or gene modification on survival, development, behavior, pathology, etc.

http://sgugenetics.pbworks.com/w/page/24704977/Creation%20of%20a%20%22Knockout%20Mouse%22
Confirming success of RPE65 Knockout

Has the “new” sequence replaced original gene? (GENOTYPING)
- Southern blot of genomic DNA
- PCR amplify altered and normal genes
- Sequence to verify

Do homozygous knockout animals lack the normal RPE65 mRNA and protein?
- Purify RNA and Protein from RPE of normal and knockout animals
- For RNA: northern and/or RT-PCR
- For protein: western and/or immunostaining

Does the phenotype correspond to human LCA?
- Analyze retinal function and structure over time in knockout and normal animals
  - ERG, OCT, histology
- Interaction with other genes? Breed to other mutant strains

Develop and test gene replacement drugs
Applications of PCR: Genotyping

PCR with specific primers to amplify target sequences transgenic modification

Normal allele

Mutant allele (knockout)

152 bp

139 bp contains deletion
Agarose Gel Electrophoresis

2. DNA segments are loaded into wells in a porous gel. The gel floats in a buffer solution within a chamber between two electrodes.

3. When an electric current is passed through the chamber, DNA fragments move toward the positively-charged cathode.

4. Smaller DNA segments move faster and farther than larger DNA segments.

http://www.stanford.edu/group/hopes/diagnosis/gentest/f_s02gelelect.gif
Applications of PCR: Genotyping Knockout Mouse

- Gel electrophoresis of PCR fragments to identify mice with and without mutant allele
  - Heterozygotes carry both alleles
  - Homozygous transgenic mice have only mutant allele

PCR products

<table>
<thead>
<tr>
<th>Normal allele</th>
<th>Mutant allele (knockout)</th>
</tr>
</thead>
<tbody>
<tr>
<td>152 bp</td>
<td>139 bp</td>
</tr>
</tbody>
</table>

Gel image

<table>
<thead>
<tr>
<th>mouse #</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
</tr>
<tr>
<td>7</td>
</tr>
<tr>
<td>6</td>
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</tbody>
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+/KO  +/+  KO/KO
Is *RPE65* RNA absent in KO mouse?
RT-PCR (Reverse Transcription PCR)

- A method to analyze RNA:
  - Used to analyze gene expression
    - Determine levels of different transcripts in sample
    - Identify tissues/cells that express gene
    - Developmental timing of gene expression
    - Look for alternative splicing (different primers for different splice forms)
  - Can also be used to generate specific cDNAs for cloning

- Strategy
  - Isolate RNA from tissue/cells of interest
  - Reverse transcribe (RT) RNA into cDNA
  - Design gene-specific primers based on known sequence
  - Include control reactions for known RNA that should still be present in KO
  - PCR amplify target
  - Gel electrophoresis
  - Sequence to verify correct gene is amplified
  - May need to clone PCR product into plasmid or virus depending on next use
Qualitative RT-PCR Analysis of Gene Expression

1. Isolate RNA from normal (long arrows) and KO (short arrows) animals
   - brain (#1, 2)
   - retina (#3, 4)
   - RPE (#5, 6)

2. Reverse transcribe (RT) RNA into cDNA

3. RT-PCR using primers for different genes (each panel shows a different gene)

4. Separate PCR products on agarose gel

5. Stain with ethidium bromide

6. Presence of band = mRNA present in sample

Agarose gel of RT-PCR products

Gene F = RPE65
- Present in RPE from normal mouse #6
- Absent in RPE from KO mouse #5
- Absent from retina and brain in KO (#1, 3) and normal (#2, 4) mice
Is RPE65 protein absent in KO? Immuno-histochemistry / Immuno-fluorescence

**Purpose:** Determine the cellular expression of protein within a tissue, cell or organism

**Sample:** target tissue or cells; usually fixed to preserve structure

**Diagram 1:** Illustration of Indirect Immunohistochemistry and Immunofluorescence methods.

**Experimental Procedure:**

**Probe:** Antibodies that recognize specific protein (primary antibody)

**Detection:** Tagged antibodies that recognize primary antibody (secondary antibody)
Example of Immuno-histochemistry & Immuno-fluorescence of Protein Expression

- **Immunohistochemistry**: secondary tagged with enzyme; apply substrate for colorimetric detection.
- **Immunofluorescence**: secondary antibody tagged with fluorescent molecule

- Section from retina
- Stained for 2 different proteins
  - immuno-fluorescence (red)
  - immunohistochemistry (brown)
- Arrows show double-labeled cell containing both proteins

Note: example illustrated is NOT RPE65
Analysis of *RPE65* knockout mouse

Study function and disease association

Comparison of Ocular Pathologies in Vitamin A–Deficient Mice and RPE65 Gene Knockout Mice

Yang Hu, Ying Chen, Gennadiy Moiseyev, Yusuke Takahashi, Robert Mott, and Jian-xing Ma

Investigative Ophthalmology & Visual Science, July 2011, Vol. 52, No. 8
Copyright 2011 The Association for Research in Vision and Ophthalmology, Inc.

Entainment of circadian rhythm to a photoperiod reversal shows retinal dystrophy in *RPE65*\(^{-/-}\) mice

D.M. Daniels\(^{a, b}\), C.W. Stoddart\(^{a, b}\), M.T. Martin-Iverson\(^{b, c}\), C.-M. Lai\(^{d}\), T.M. Redmond\(^{d}\), P.E. Rakoczy\(^{d}\)


Cone opsin mislocalization in Rpe65\(^{-/-}\) mice: a defect that can be corrected by 11-cis retinal.

Rohrer B\(^{1}\), Lohr HR, Humphries P, Redmond TM, Seeliger MW, Crouch RK.
Goals & Problems for Gene-based Therapies

• **Goal: Treat the underlying cause of the disease**
  – Stop pathological processes
  – Eliminate malfunctioning proteins
  – Replace missing metabolites/proteins/genes
  – Add genes/cells that will make corrected and/or therapeutic proteins

• **Problems: Delivery and Specificity**
  – Target affected cells
  – Reduce unwanted side-effects

• **Problems: Sustainability**
  – Eliminate need for frequent injections/drops/pills
  – Minimize need for repeated surgical interventions
## Developing Gene Therapy for RPE65

- Identify method to deliver gene to target cells
  - Often uses viral vectors
  - Viral genome must be large enough to contain entire RPE65 coding region and appropriate promoter
  - Preferably doesn’t deliver into other cell types
  - Minimal immune response

- **CLONE RPE65 Genes**
  - **PCR amplify cDNA** for full length gene from appropriate species
  - **Sequence to confirm that sequence of clone is perfect**
  - Identify appropriate **promoter** to drive expression
    - Must be specific for RPE cells
    - Must be active to drive high levels of transcription of the therapeutic gene

- Use restriction enzymes to clone RPE65 cDNA and promoter into viral genome
  - Package modified viral genome into virus
  - Typically eliminate viral sequences to prevent replication of virus in RPE cells

- Subretinal injection to apply virus next to RPE
  - Functional testing
  - Animal models (small: mouse, rat; large: dog)
Somatic Cell Gene Therapies Delivery

- Package cDNA and promoter for normal RPE65 gene in viral vector
  - (for this application, used AAV=adeno associated virus)
- Virus infects cells-sends DNA to nucleus
- New gene inserts into genome and makes correct gene product

Human Gene Therapy Retinal Degeneration: Strategy: Replace mutant/missing gene

1. Healthy eye
   In a healthy eye, retina cells at the back detect light

2. Cells dying
   A faulty gene means these cells are dying, leaving all but a small section - causing partial blindness

3. Lift retina
   To stop further degeneration, fluid is injected to lift a layer of the cells

4. Inject DNA
   Working copies of the faulty gene are then injected to stop the rest of the cells dying

From: http://www.bbc.co.uk/news/health-15446912
Preclinical: Testing Gene Therapy for LCA/RPE65

Gene Therapy Restores Vision-Dependent Behavior as Well as Retinal Structure and Function in a Mouse Model of RPE65 Leber Congenital Amaurosis

Ji-jing Pang,1,* Bo Chang,2 Ashok Kumar,3 Steven Nusinowitz,4 Syed M. Noorwez,1 Jie Li,3 Asha Rani,3 Thomas C. Foster,3 Vince A. Chiodo,1 Thomas Doyle,1 Huashi Li,1 Ritu Malhotra,1 Jacqueline T. Teusner,1 J. Hugh McDowell,1 Seok-Hong Min,1 Qiuhong Li,1 Shalesh Kaushal,1 and William W. Hauswirth1

Gene therapy restores vision in a canine model of childhood blindness

Gregory M. Acland, Gustavo D. Aguirre, Jharna Ray, Qi Zhang, Tomas S. Aleman, Artur V. Cideciyan, Susan E. Pearce-Kelling, Vibha Anand, Yong Zeng, Albert M. Maguire, Samuel G. Jacobson, William W. Hauswirth & Jean Bennett

Nature Genetics 28, 92–95 (2001) | Download Citation
Results at 5 Years After Gene Therapy for RPE65-Deficient Retinal Dystrophy.


Safety and efficacy of gene transfer for Leber's congenital amaurosis.


Age-dependent effects of RPE65 gene therapy for Leber's congenital amaurosis: a phase 1 dose-escalation trial.


Gene therapy for leber congenital amaurosis caused by RPE65 mutations: safety and efficacy in 15 children and adults followed up to 3 years.

Identify patients with mutations in *RPE65*: Genetic Testing

- The *RPE65* gene is 1 out of more than 260 genes that may be responsible for inherited retinal disease
- Approved gene therapy only works for patients with RPE65 mutations

- Differential diagnosis
- Informed consent
- Collect sample (saliva or blood)
- Send to company
  - DNA isolation
  - Microarray analysis
  - ~2 weeks to get genetic report
- Referral to approved gene therapy or ongoing clinical trials as appropriate

The *RPE65* Genetic Testing Guide

Ordering genetic tests to confirm the presence of biallelic *RPE65* gene mutations—the first step to identifying appropriate patients for LUXTURNA® (voretigene neparvovec-rxyl)

Resources for more information

Good discussion/information resource for gene therapy, DNA testing, stem cells, and more. Not overly technical but very informative.

https://www.risingtidebio.com/

Info on methods:

https://www.thermofisher.com/us/en/home/brands/invitrogen/molecular-biology-technologies/mol-bio-school.html?gclid=EAIaIQobChMIhPOUs-Gq5AIVif_jBx0Dzgs-EAMYASAAEg1eFvD_BwE&ef_id=EAIaIQobChMIhPOUs-Gq5AIVif_jBx0Dzgs-EAMYASAAEg1eFvD_BwE:G:s&s_kwcid=AL!3652!3!285361324789!p!!g!!molecular%20biology%20technique

https://www.researchgate.net/publication/226072152_Basic_Techniques_in_Molecular_Biology