A Survey of Genetic Methods
DNA-RNA Hybridization
**reverse transcriptase – polymerase chain reaction**

1 copy → Heat to 95°C to denature DNA. Cool to 37°C to allow hybridization of primers to DNA

2 copies → Taq polymerase extends complementary strands from primers

First synthesis cycle results in two copies of target DNA sequence

Second cycle

Denature DNA

Hybridize primers

Extend new DNA strands

Second synthesis cycle results in four copies of target DNA sequence

RT-PCR
DNA Microarrays

Expression arrays compare messages from different cell types or from cells at different developmental stages.

PREPARE cDNA “TARGETS”

Cell type 1

RNA

Reverse transcription, label with fluorescent dyes

Cell type 2

mRNA from cell 1 labeled with fluorescein (green fluorescence)

mRNA from cell 2 labeled with rhodamine (red fluorescence)

Combine equal amounts
Microarrays, cont.

Probe cDNAs made from a given tissue; “printed” on slide or membrane

PREPARE MICROARRAY “PROBES”

Hybridize target to microarray

Scan

green - mRNA is more abundant in cell type 1

yellow - mRNA equally abundant in both cell types

red - mRNA more abundant in cell type 2
DNA Macroarrays

Essentially the same technology as microarrays, but larger; analysis by fluorescence or autoradiography (targets labeled with radioactive tags).
In Situ Hybridization

Anti-sense mRNA probe with fluorescent or radioactive label hybridized with message contained within the fixed tissue.

Whole-mount in situ hybridization:
e.g. detection of digoxigenin-labeled probe using alkaline phosphatase-conjugated antibody

1. RNA made complementary to specific mRNA
2. Digoxigenin label on uridine
3. Hole in cell membrane
4. mRNA
5. Alkaline phosphatase-conjugated antibody to digoxigenin
6. Alkaline phosphatase
7. Colorless compound that becomes purple dye when phosphate is removed
8. Wash
9. Wash
Reporter Genes

Construct gene with enhancer element of choice attached to structural gene for reporter molecule
e.g. Pax6 enhancer elements attached to β-galactosidase gene

Enhancers: A B C D
Exons: 0 1 2 3 4 5 5a 6 7

mouse – Pax6

LacZ – β-galactosidase

Pax6 exons

add a gene for reporter protein, e.g. β-galactosidase (blue) luciferase (produces light) green fluorescent protein (GFP)
Transgenic Cells and Organisms

Methods for inserting foreign DNA into a cell

1. Microinjection

2. Transfection
   - lipophilic agents
   - form transient membrane openings (nm sized)

3. Electroporation
   - electric pulses transiently disrupt membrane

4. Transposable elements
   - endogenous mobile DNA regions
   - artificially insert genes of interest

5. Retroviral vectors
   - RNA-containing viruses
   - reverse-transcriptase – cDNA
     - incorporates into host DNA
   - insert gene of interest into existing retroviral sequence

6. P elements (Drosophila)
   - family of endogenous transposable elements
   - insert gene of interest
Embryonic Stem Cells

mammalian blastocyst

inner cell mass
- embryo proper

trophoblast
- embryonic contribution to the placenta

Embryo donor parent mice

Embryonic stem cell

Inner cell mass

Trophoblast

Culture of embryonic stem cells
Chimera Formation

mammalian blastocyst

inner cell mass
- embryo proper

- embryonic contribution to the placenta
trophoblast

Embryo donor
parent mice

Embryonic stem cell

Inner cell mass

Trophoblast

vector contains antibiotic resistance gene

insert gene of interest into embryo using
- transfection
- microinjection
- etc.

Culture of embryonic stem cells

Mix embryonic stem cells with cloned gene
Chimera Formation

NOTE –
growth medium contains antibiotic!

ES cells (with transgene and antibiotic resistance gene)

Select those embryonic stem cells that have incorporated the transgene

Microinject transgenic ES cells into host embryo

Transgenic ES cells integrate into embryo - and some may integrate into the germ line (gamete progenitors)

Inject embryos into uterus
Chimera Formation

Inject embryos into uterus

Chimeric progeny mice

Cross chimeric mouse and normal mouse

Chimeric

Wild-type

Heterozygous chimeric
Chimera Formation

Cross heterozygous progeny

Heterozygous transgenic mice

Homozygous wild-type (25%)

Homozygous transgenic (25%)

Heterozygous transgenic (50%)

mouse (R) - transgenic for human growth hormone

R. L. Brinster and R. E. Hammer
Gene Knockouts

**Targeted gene insertion**
- similar to chimeric method, but
- selectively replaces functional genes with non-functional genes

e.g. target gene: bone morphogenic protein 7 (BMP7)

mutation: insert a gene for neomycin resistance into the BMP7 gene

- $\text{neo}^r = \text{neomycin resistance}$
Gene Knockouts

Targeted gene insertion
- similar to chimeric method, but
- selectively replaces functional gene with non-functional gene

e.g. target gene: bone morphogenic protein 7 (BMP7)
mutation: insert a gene for neomycin resistance into the BMP7 gene

NOTE – the efficiency of homologous recombination is very low; very few genes will be properly incorporated
Gene Knockouts

Select heterozygous ES cells by their neomycin resistance

Inject heterozygous ES cells into blastocyst

Inject blastocysts into uterus

Formation of chimeric mice

The presence of the antibiotic-resistance gene allows selection of cells that successfully incorporated the target gene.
Gene Knockouts

Breed chimeric to wild type

Mate heterozygous $Bmp7^{+}/Bmp7^{-}$

Wild-type $Bmp7^{+}/Bmp7^{+}$

Heterozygote $Bmp7^{+}/Bmp7^{-}$

Heterozygote $Bmp7^{+}/Bmp7^{-}$

Wild-type $Bmp7^{+}/Bmp7^{+}$

Homozygote $Bmp7^{-}/Bmp7^{-}$

Heterozygote $Bmp7^{+}/Bmp7^{-}$
Antisense RNA

mRNA – single stranded message = “sense” strand: 5’C-A-U-G3’
complement (natural or synthesized) = “antisense” strand: 3’G-U-A-C5’

Insertion of antisense RNAs into the cell blocks gene expression:
- translational blockage (synthetic antisense RNAs)
- transcriptional blockage (postulated function of natural antisense RNAs)

Methods
- synthesis and insertion of antisense RNA
  - binds to native mRNA
double stranded RNA subject to enzymatic destruction (Dicer nuclease)
- morpholino antisense oligomers
  - morpholino molecules used in strand construction
  - morpholino oligomers do not degrade;
    effective over several cell generations

Note - naturally-occurring antisense RNAs also exist:
- paternal gene for IGF2r receptor contains antisense sequence
- blocks synthesis of IGF2r
RNA-Interference

RNA interference (RNAi)
- double-stranded RNAs inserted;
  - short interfering RNA (siRNA)
- leads to the degradation of native mRNA
- RNAi is common in plants, animals, fungi
  - probably evolved as a defense against retroviruses = dsRNAs

**Dicer function**
- Dicer enzyme complex binds to dsRNA
  - cleaves into small interfering RNA (siRNA)
  - Note - when dsRNA is from endogenous source = microRNA (miRNA)

**RISC** - RNA-induced silencing complex
- argonaute component of Dicer complex catalyzes mRNA degradation
RNAi Knockdown

RNAi-inhibited transcription: “knockdown” experiments
- blocks transcription of specific genes
  - lack of gene = lack of protein = lack of function
- advantage: effect can be seen at certain time in certain tissue
- like “knockouts, but not as complete
  - siRNAs specific for particular sequence
  - miRNAs less specificity

Native RNAi triggers:

siRNA – dsRNA, probably from RNA viruses

microRNA (miRNA) - genetically coded sequences; non-coding; control some translation during portions of development; undergo extensive post-transcriptional modification

\[
\begin{array}{c}
\text{U} \\
\text{U} \\
\text{C} \\
\text{U} = \text{A} \\
\text{C} = \text{G} \\
\text{U} = \text{A} \\
\text{C} = \text{G} \\
\text{C} = \text{C} \\
\end{array}
\]

5’ CCAAAG = CACCAA 3’

miRNA stem-loop structure

aka “short hairpinRNA”

shRNA
Developmental Methods

Concept: Genomic Equivalence

Methods:

Cloning
  - amphibian
  - mammalian

Embryonic stem cells
Concept: Genomic Equivalence

If every cell in an organism has the same set of genes (genomic equivalence), shouldn’t a nucleus from one cell be able to direct the development of any other cell, even in the zygote?

Test: somatic nuclear transplantation (cloning)

Technical challenges:
- enucleation of a recipient egg
- acquisition of competent donor nuclei
- ability to transfer nuclei into enucleated oocytes
Amphibian Cloning

1. Extract and lyse donor cell
2. Use Micropipette to insert Donor nucleus into enucleated cell
3. Membrane heals
4. Somatic cell nucleus in an activated egg
5. Animal pole
6. Glass needle
7. Egg cell membrane
8. Vitelline envelope
9. Meiotic spindle
10. Remove chromosomes and spindle from cell
11. Activated enucleated egg
12. Isolated meiotic spindle
Effect of Age in Nuclear Transplants

Transplant nuclei from different aged donors....

Rana pipiens

Loss of ability of nuclei to support development when transferred demonstrates a loss of blastomere potency as development proceeds.
Serial Transplantation

Clones made from differentiated nuclei died shortly after the blastula stage. However, if blastomeres from those blastulae were used as nuclear donors.... ...and the process was repeated several times.... clones could survive.

*Xenopus laevis*

Although tadpoles died prior to feeding, the experiment demonstrates that differentiated nuclei still had pluripotent potential.
Mammalian Cloning

OOCYTE DONOR

Eggs removed

Meiotic spindle

Remove spindle

Enucleated egg

NUCLEAR DONOR

Udder cells removed

Udder cells grown in G₁ stage.

Transfer cell into enucleated egg

- differentiated cell
Mammalian Cloning – 2

- Inner cell mass (ICM) – forms embryo proper
- Trophoblast – forms connections with placenta

Egg and cell fused

Embryo cultured 7 days

Electroporation - destabilizes cell membranes; allows fusion

Mammalian cloning – success in multiple species, however...
- Inefficient (Dolly = 1/434 transfers)
- Problems
  - Large fetus
  - “Old” chromosomes
  - Disease susceptibility
  - Premature aging