Transgenic animals

Transgenic animals

Animals which have been genetically engineered to contain one or more genes from an exogenous source.

A large number of transgenic animals have been created.
Mice, Cows, Pigs, Sheep, Goats, Fish, Frogs, Insects

Knockout animals why?

- To study effects of gene products, biochemical pathways, alternative (compensatory) pathways, and developmental pathways.

- To recreate human diseases in animals to establish models to test the beneficial effects of drugs or gene therapy.

Procedure for Generating Knockout Mouse

- Gene alteration in KO mice is targeted to very specific genes.
- DNA must integrate at precise positions in the genome.
- Integration of the altered gene takes place in embryonic stem cells ex vivo.
- Verification of exact location of integration occurs before the ESC is introduced into blastocysts to become part of the developing embryo.

Introduction of foreign genes into intact organisms

Main ways to produce transgenic animals:

- Microinjection
- Embryonic stem cell (ES) based transgenesis,
  ES cell injection into blastocyst
- Using retrovirus

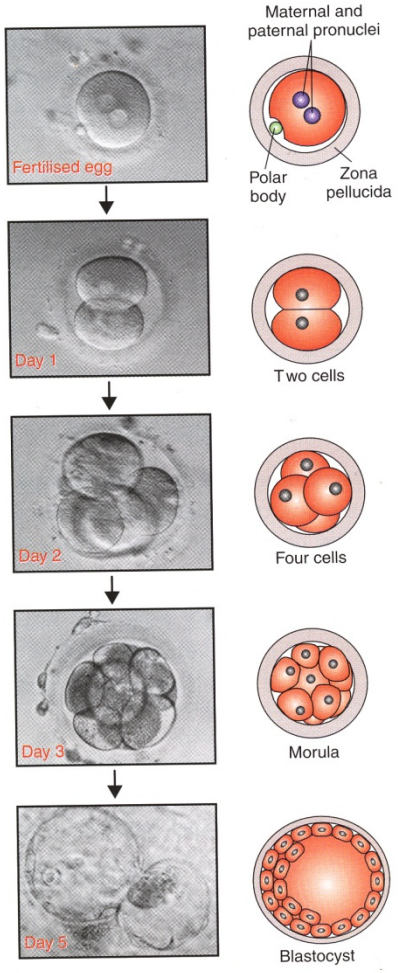
Early embryonic development

When sperm enters the egg, the fertilized cell is called ZYGOTE, it contains two nuclei – PRONUCLEI

Maternal and paternal pronuclei fuse with each other to form a single fertilized nucleus.

Zygote then starts to divide into 2, then 4, 8, and more forming a ball of cells – MORULA

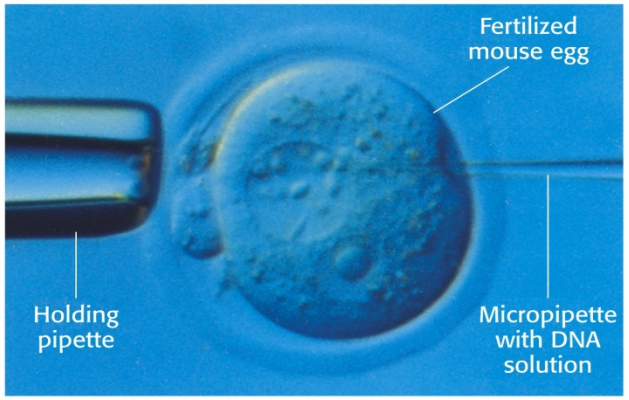
Morula continues to divide and the cavity is filled with the fluid from uterus. It is called BLASTOCYT in this stage.



Pronuclear Injection

The injection into the nuclei of newly fertilized eggs

- The eggs are harvested from mice (superovulated or natural matings).
- Following fertilization, large male and small female pronuclei are visible under the microscope.
- DNA injections are usually made into the larger male pronucleus and the while egg is held using pipette.



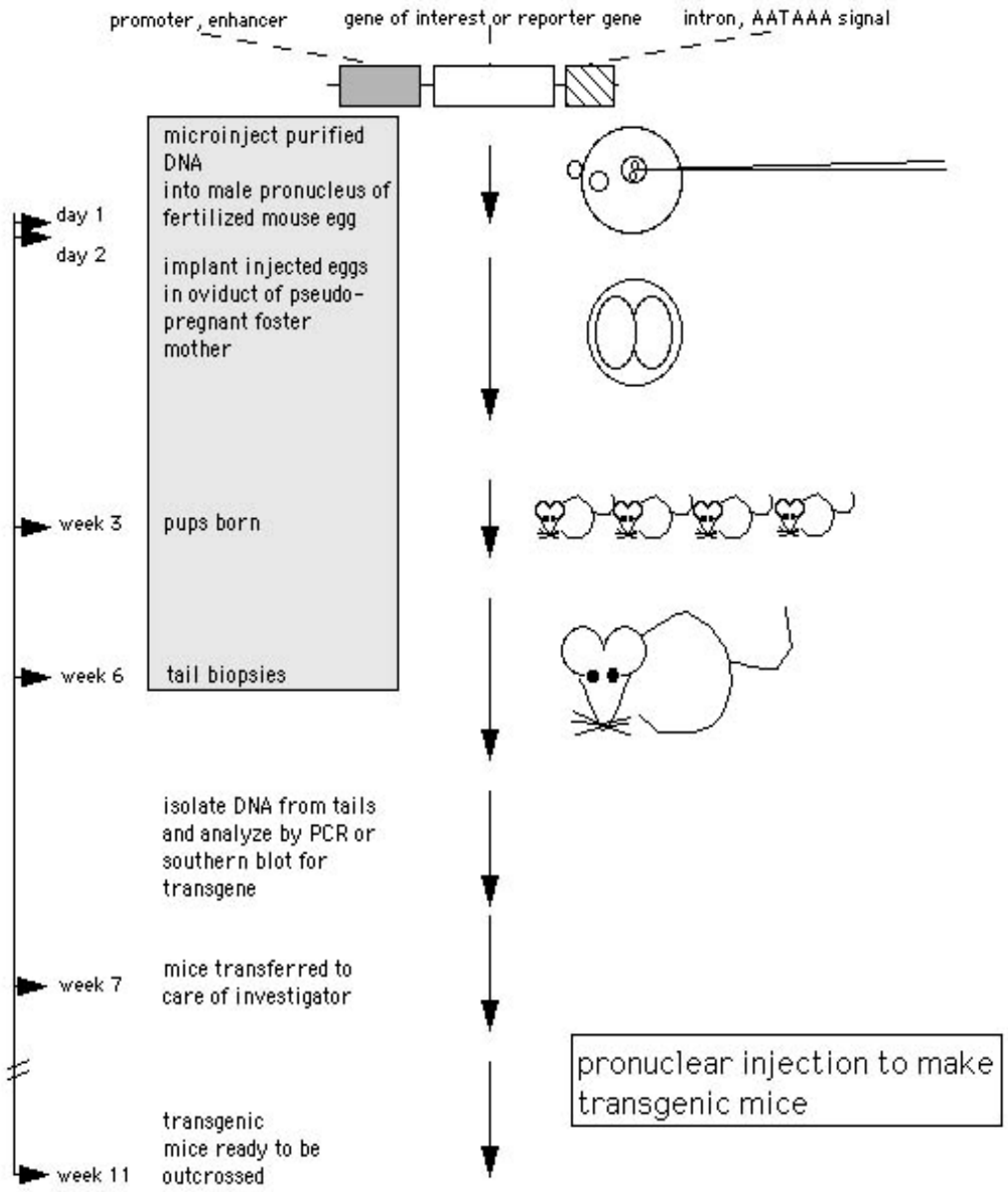
Gene is injected into the male pronuclei

Pronuclear Injection

The injected DNA is integrated into the pronuclear DNA and upon fusion with the female pronucleus, is incorporated into zygote.

The injected embryos are cultured *in vitro* until the morula stage and then implanted into the pseudo-pregnant female mouse **that has been previously mated with a vasectomized male, which stimulated the appropriate hormonal changes needed to make her uterus receptive.**

The implanted embryo is then allowed to develop into a mouse pup.



Mechanisms of DNA Integration

- Linear molecules integrate more efficiently than circular molecules (~5x).
- Once in the oocyte, the linear molecules circularize.
- Usually all of the molecules that integrate are on the same chromosome and at the same site.
- Multiple copies are usually arranged in a tandem, head-to-tail array.
- The size of the DNA molecule (0.7 – 50Kb) is not an important parameter.
- The concentration and purity of the injected DNA is critical (1-3 μg/ml maximum).

Application of Pronuclear Injection

Variety of foreign DNA were introduced into mice:

This technique has been used to attempt to produce therapeutic proteins, e.g. human α-antitrypsin (AAT) for treatment of cystic fibrosis.

A DNA fragment containing the human ATT gene, whose promoter was replaced by that from the sheep β -lactoglobulin milk promoter was injected into the pronucleus. The transgenic Mouse expressed ATT in the mammary gland secreted into its milk.

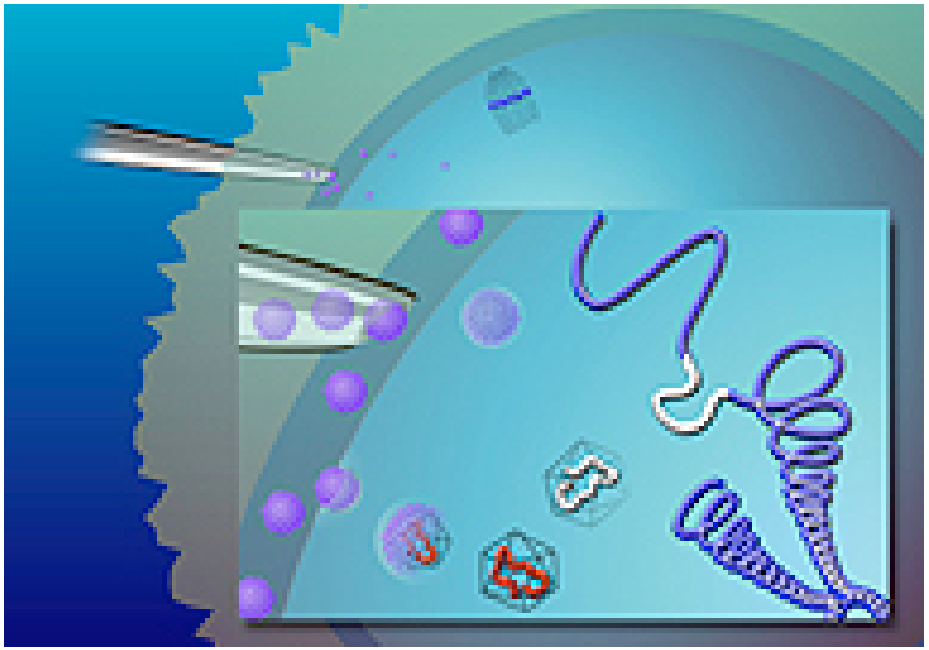
The transient sheep expressing ATT in their milk have been produced in the same way.

Disadvantages

- Pronuclear injection cannot be used for knock-out genes or to alter existing genes.
- Randomness of the insertion can dramatically effect the expression of the foreign gene. The expression of the transgene cannot be readily controlled.
- The transgene can be present in a limited set of tissue and organs, when integration of the transgene is delayed after the first cell division.
- More than one copy of the gene may get into the genome.

Lentiviral infection

Lentiviral based vector

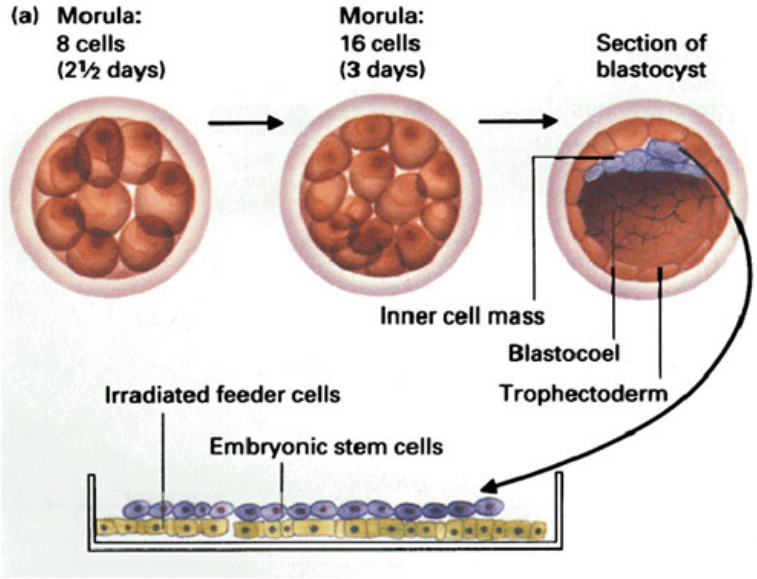


Eggs are infected prior to fertilization

Virus integrates into one of the chromosomes, non specific integration

Blastocyst Injection and Embryonic STEM Cells

- This involves placing the transgene into an embryonic stem cells (ES cells).
- ES cells have the potential to form all the cell types (muscle, nerve, skin, gametes= are pluripotent.
- ES cell can be cultured *in vitro* in a dish coated with mouse embryonic skin cells that do not divide (feeder layer).
- Feeder layer provides a surface for attaching ES cells and releases nutrients in the medium.
- ES cells in the culture are not differentiated during cultivation, they grow separately.
- When clump together, they begin spontaneously differentiate.



Production of transgenic mice by ES cell gene transfer

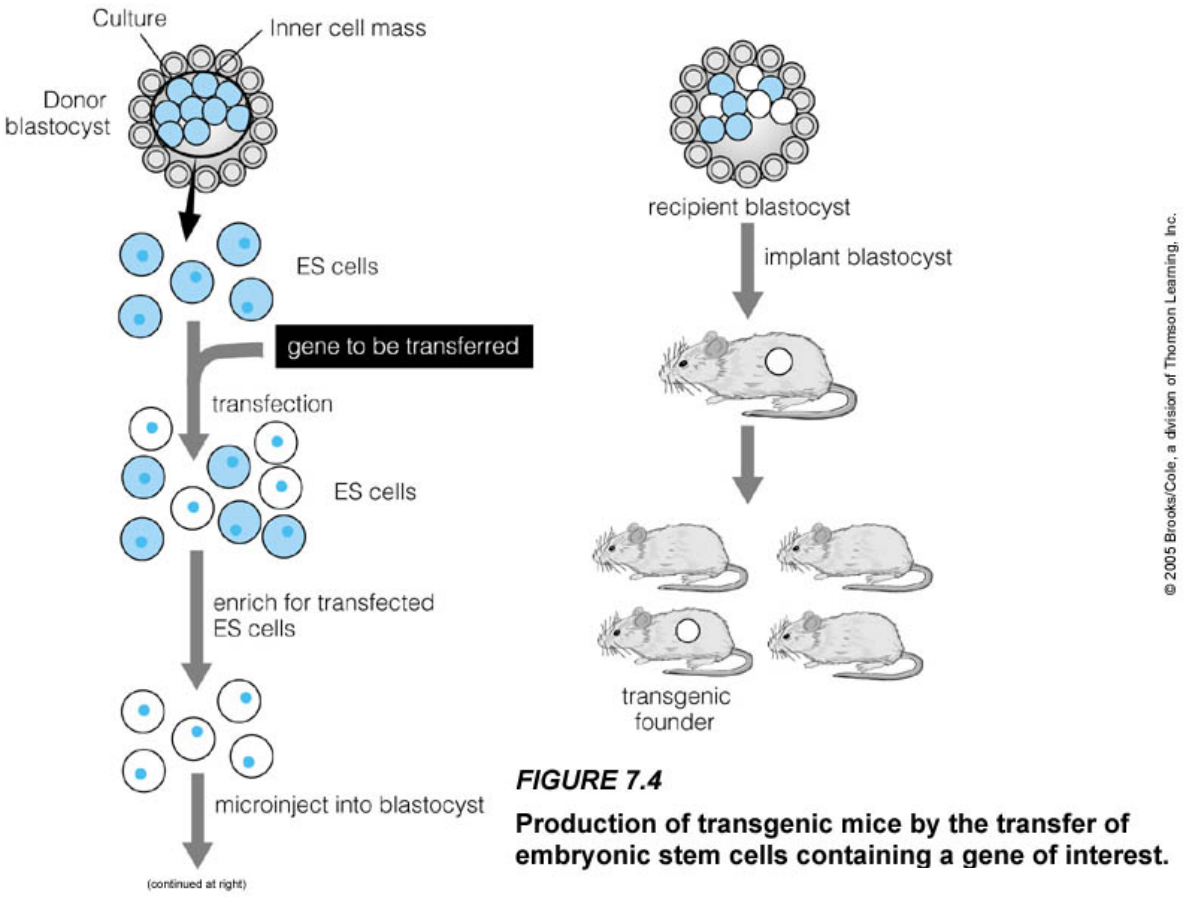


FIGURE 7.4

Production of transgenic mice by the transfer of embryonic stem cells containing a gene of interest.

The blastocyt is implanted into the uterus of a pseudopregnant female and pups are produced.

The implanted blastocyst contains two different ES cells (normal and recombinant).

The resulting offspring will be chimeric = some cells will contain transgene, other not.

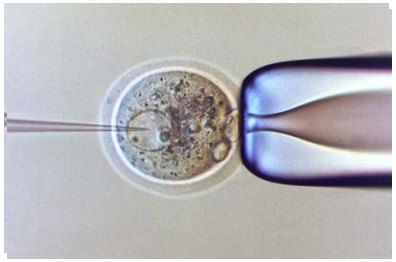
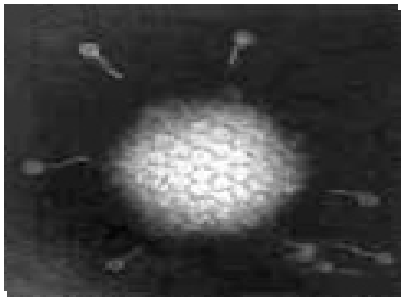
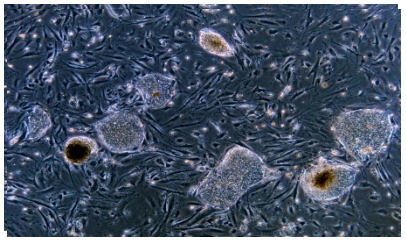
The chimeric pups are crossed with the wild type animals to generate true heterozygotes, which can be subsequently inbred to create a homozygote.

Advantages using ES Cells

- Embryonic stem cells are relatively efficient at homologous recombination.
- Recombination between homologous sequence in the vector DNA and the genome is used to target the insertion of the foreign DNA to a specific sequence in the genome.
- Some levels of non-homologous recombination occur.
- It is necessary to separate these two types.

- This method allows also to delete (knockout) a gene.

Transgenesis Methods

	pros	cons
Pronuclear injection 	Relatively simple and efficient Long transgenes possible Potentially all species	Random integration Multicopy insertions (Strain limitations)
Lentiviral infection 	Very efficient Single copy insertions No technical equipment Works in many species	High embryo mortality 9.5 kb packaging limit Safety issues (?) Only random integration
ES based transgenesis 	Long transgenes possible Gene targeting possible Single copy insertions	Technically difficult Time consuming Species / Strain limitations

Mutagenesis is not possible by these techniques

Gene Editing Methodologies

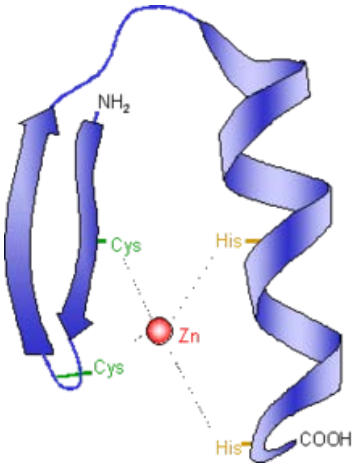
- Gene editing is a type of genetic engineering that utilizes engineered nucleases to bring about targeted alterations at specific sites within the DNA. Three gene editing methodologies presently being used include Zinc Finger Nucleases (ZFNs), Transcription activator-like effector (TALE) nucleases and CRISPER.

- DNA is inserted, replaced, removed from a genome using artificially engineered nucleases that create double stranded break and activate cell system to repair the break by homologous recombination (HR) and non-homologous end –joining (NHEJ).
- Nucleases can cut any targeted position in the genome

Zinc finger nuclease (ZFN) and TALEN

ZFNs – artificial restriction enzymes consisting of DNA recognition - Zn finger domains and DNA-cleavage domain - FOKL nuclease

DNA binding domain:
Several Zn fingers with different specificity for DNA basepairs are usualy fused to recognize larger DNA sequence



Dimeriatization of Folk nuclease induces cleavage of DNA.

ZNF

Sigma-Aldrich

ZNF technology requires two constructs with unique DNA binding domains for sites in the target genome with proper orientation and spacing fused to FokI.

For new target sequence developing new DNA binding domain.

DNA cleavage stimulates the cell's natural DNA-repair processes, namely homologous recombination and Non-Homologous End Joining (NHEJ)

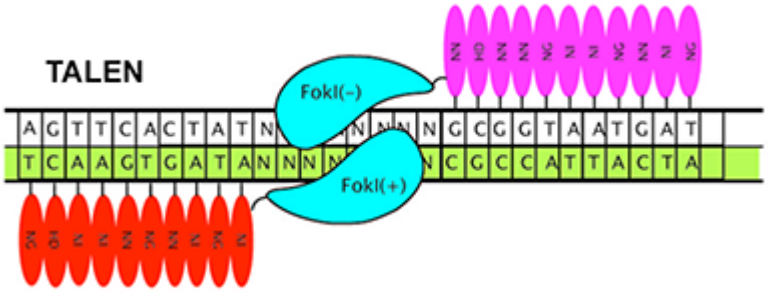
TALENs

Transcription activator-like effector (Tal): produced by plant pathogenic *Xanthomonas* bacteria - activate plant gene expression

Tal effector contains repeats of a 33-35 amino acids with divergent 12th and 13th amino acids. These two positions, referred to as the Repeat Variable Di-residues (RVD), are highly variable and show a strong correlation with specific nucleotide recognition.

Talen can be mutated to generate sequence specific binding by selecting a combination of repeat segments containing the appropriate RVDs.

TALEN technology:
fusion of a TAL effector DNA-binding domain to a DNA cleavage domain FokI, which cleaves host DNA between the two distinct TAL array binding sites.



New DNA fragment can be ligated at this site by homologous recombination.

Engineering of TALEN system

TALE genes can be mutated to generate sequence-specific DNA binding proteins

The modified TALEs can be fused to nucleases for targeted DSBs in plants and animals

Modified TALEs can be fused to transcriptional activators to trigger ectopic gene expression in mammals

The non-specific DNA cleavage domain from the end of the FokI endonuclease can be used to construct hybrid nucleases that are active in many different cell types.

CRISPR-Cas9 system

RNA mediated nuclease system based on interaction of RNA with DNA

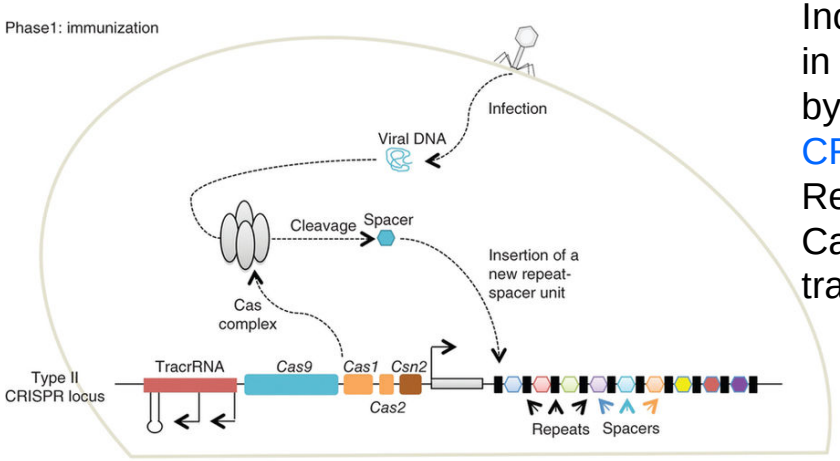
CRISPR-Cas 9 system:
Clustered regularly-interspaced short palindromic repeats and nuclease Cas

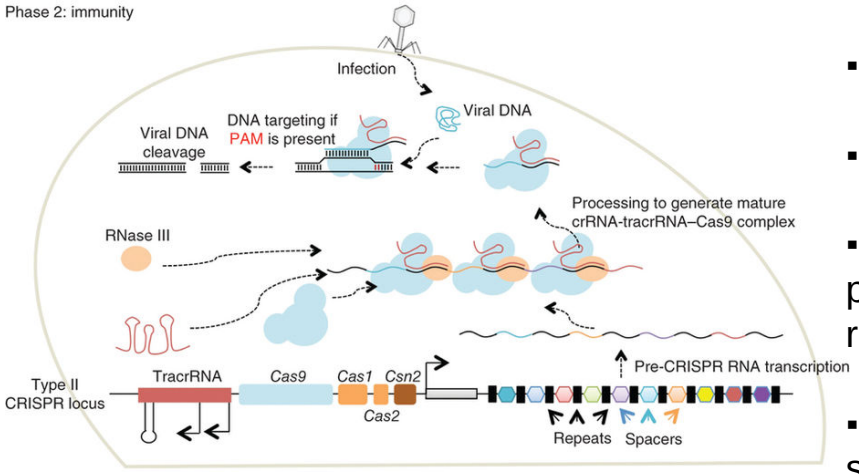
CRISPR: short (20nt) RNA recognizing ssDNA sequence

Originated from adaptive immune defense in bacteria that use short RNA to direct degradation of foreign DNA (originated from bacteriophages)

Cas 9: is an RNA-guided DNA endonuclease enzyme, which unwinds and cleaves the targetted DNA. The specificity is directed by a short guide RNA making a complex with Cas9.

CRISPR Cas 9 system in bacteria





Mali et al., Nat. Methods 10, 2013, 957.

Incorporation of viral DNA fragments in CRISPR loci as spacers separated by repeat sequences

CRISPR locus:

Repeats (28-37nt), spacers –viral DNA (32-38nt), Cas genes (nucleases and helicases), trans-activating crRNA (tracr RNA)-

- Transcription of CRISPR locus yields pre-cr RNA
- TracrRNA hybridizes to repeat region of pre-crRNA
- Endogenous Rnase III cleaves the hybridized precrRNA-tracrRNA to yield mature crRNA, which remains assocoated with tracrRNAS and Cas 9.
- This complex is specifically targetted to dsDNA sequence complementary to the spacer sequence and to protospacer-adjacent motif (PAM) and Cas9 cuts both strands.

- 3 distinct types of bacterial CRISPR systems identified so far:
  - Type I, III require more Cas nucleases
  - Type II is the basis for current genome engineering applications
    - From *Streptococcus pyogenes*
    - The *S. pyogenes* system is orthogonal to the native E. coli system

Jinek et al., Science 2012

Genomic manipulation using CRISPR-Cas 9 system

Application in other types of cells requires codon optimized Cas 9 with approopriate nuclear localization signal

cr RNA and tracrRNA expressed either individually or as a single chimera termed short guide RNA (sgRNA). Multiple crRNA and tracrRNA can be packaged together to form sgRNA.

When DNA is cleaved, it mainly gets repaired through a mechanism called Non-Homologous End Joining (NHEJ) which is a highly error prone mechanism that causes a few base pair *in*sertions or *del*etions (*indels*) at the cut site. Such an event, in most cases results in frame-shift mutation of the coding sequence, eventually leading to gene disruption (a knock-out).

In the cases where a specific mutation is to be introduced at the cut site (called knock-in), a repair template DNA is also needed as a third component of CRISPR/Cas system, that becomes inserted at the cut site through homology directed repair (HDR). The repair template DNA can be either a single stranded oligonucleotide or a double stranded plasmid/linear DNA.

Application of CRISPR-Cas9 system

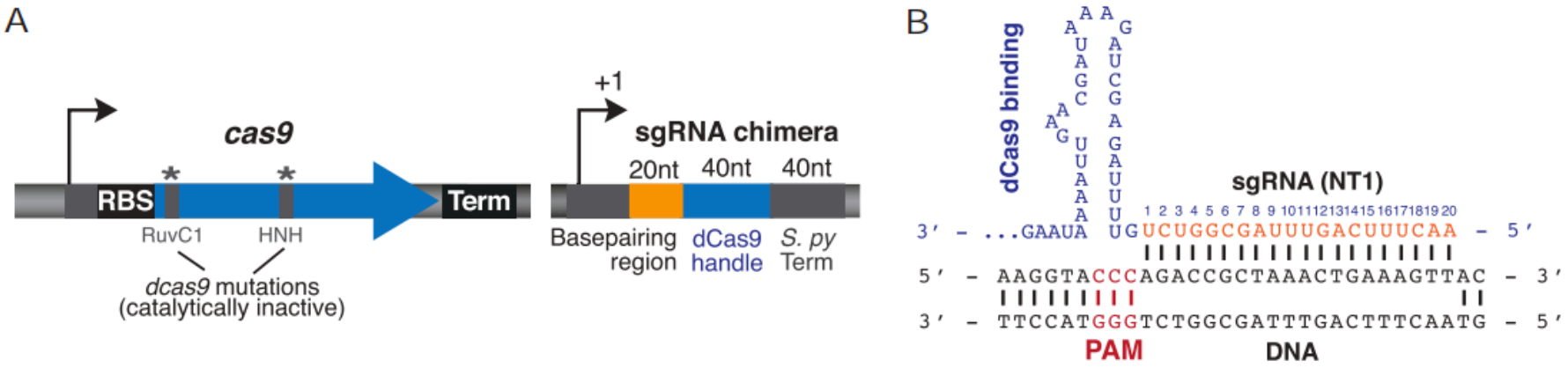
All components are delivered in one plasmid employed for cell transfection.

For new application we need to: design crRNA, repair template (overlappling with the sequences on either side of the cut and code for the insertion sequence.

The advantages of the CRISPR/Cas9 genome editing system include the ability of editing multiple genes simultaneously, a simple and fast design process that does not require the reengineering of the nuclease for each target, and an editing efficiency that is similar or greater than ZFNs and TALENs.

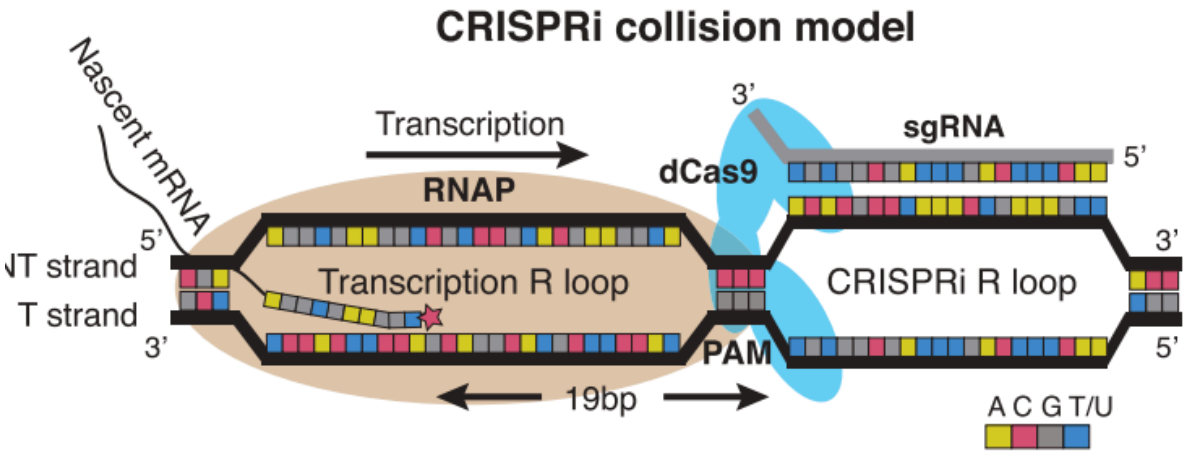
CRISPRi (CRISPR interference)

- Repurposed for genome regulation rather than gene editing
- Catalytically inactive version of Cas9 (no endonucleolytic activity)
  - Modified transcription without genetic alteration of the target gene sequence
- sgRNA comprises 3 segments:
  - 20-nt sequence complementary to target
  - 42-nt Cas9-binding hairpin (Cas9 handle)
  - 40-nt transcription terminator



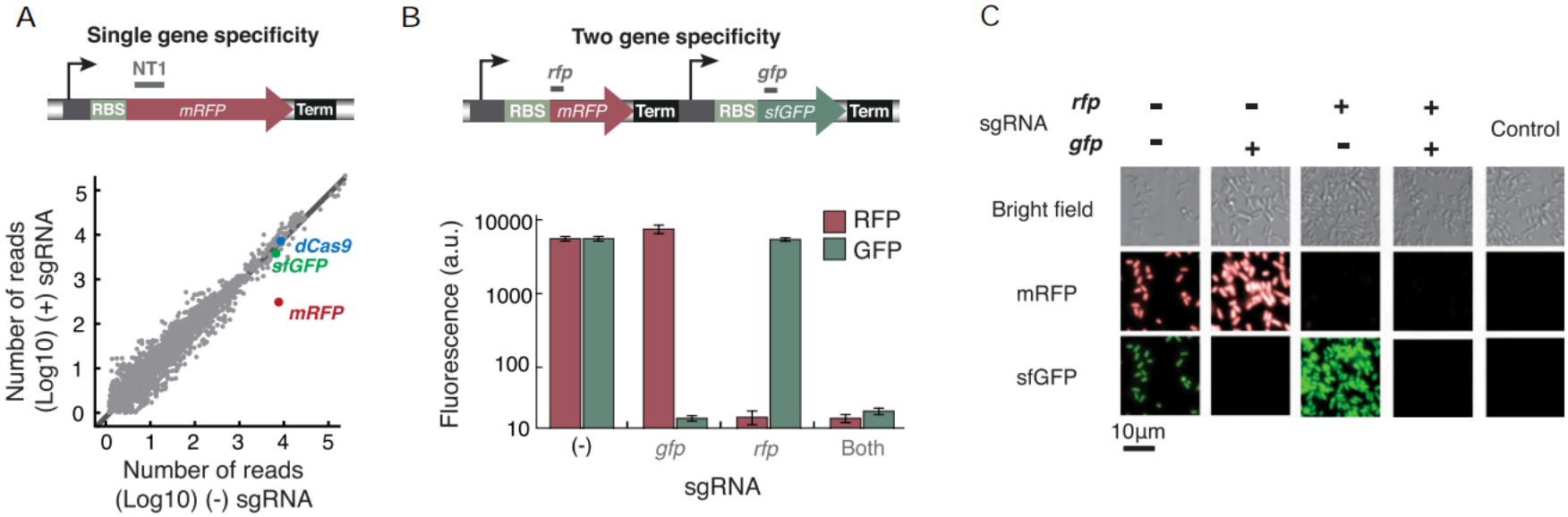
Larson et al., Nature protocols 2013
Qi et al., Cell 2013

CRISPRi functions by blocking transcription through physical obstruction of RNA polymerase

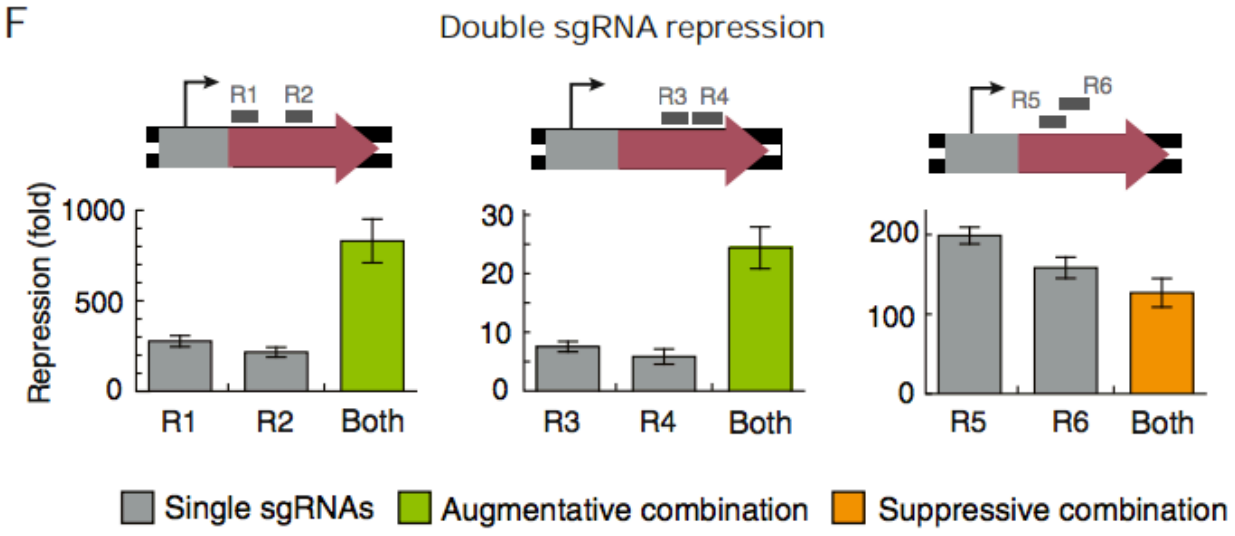


*Different from RNAi-based silencing, which requires the destruction of already transcribed mRNA

High specificity and can simultaneously regulate multiple genes



Repression efficiency can be enhanced by combining non-overlapping sgRNAs targeted against the same gene

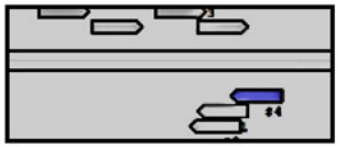
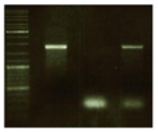
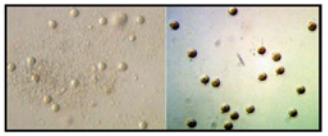
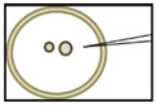
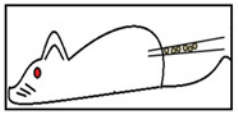
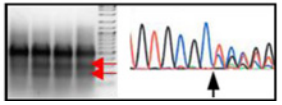


CRISPRi

- Can be used to:
  - Efficiently and selectively repress transcription of target genes
  - Target large numbers of genes to infer gene function, or map genetic interactions through gene pairs
  - Probe regulatory functions (activating / repressing / neutral) of genes and *cis* elements in a complex regulatory network
  - Manipulate microbial genomes, esp. nonmodel organisms with limited genetic tools developed
  - Image genomic loci in living cells

- Limitations:
  - The requirement for the NGG PAM sequence for Cas9 limits the availability of target sites
    - Though other Cas9 homologs may use different PAM sequences
    - *S. pyogenes* Cas9 could partially recognize an NAG PAM sequence
  - Some target sequences may be problematic due to the RNA secondary structure that may form

CRISPR- Cas mediated mouse genome editing steps

Protocol Step		Basic Protocol	Time Consideration
1) Designing of CRISPR targets		1	Week 1
2) Synthesis and purification of RNA and DNA components		2	Weeks 1 to 3
3) A. Isolation of one-cell staged embryos		3	Weeks 3 to 4
B. Micro-injection of CRISPR/Cas components into embryos		3	Week 5
C. Transferring embryos into pseudoprgenant mice		3	Week 5
4) Genotyping of offspring.		4	Weeks 9-11

Harms et al. Curr Protoc Hum Genet. 2014; 83: 15.7.1–15.7.27.

Transgenic Animals: Potential Problems

- Technical problems to closely mimic a desired situation
- Underestimation of biological complexity
- Mouse – Human differences
- Inappropriate analysis
- Undefined genetic backgrounds