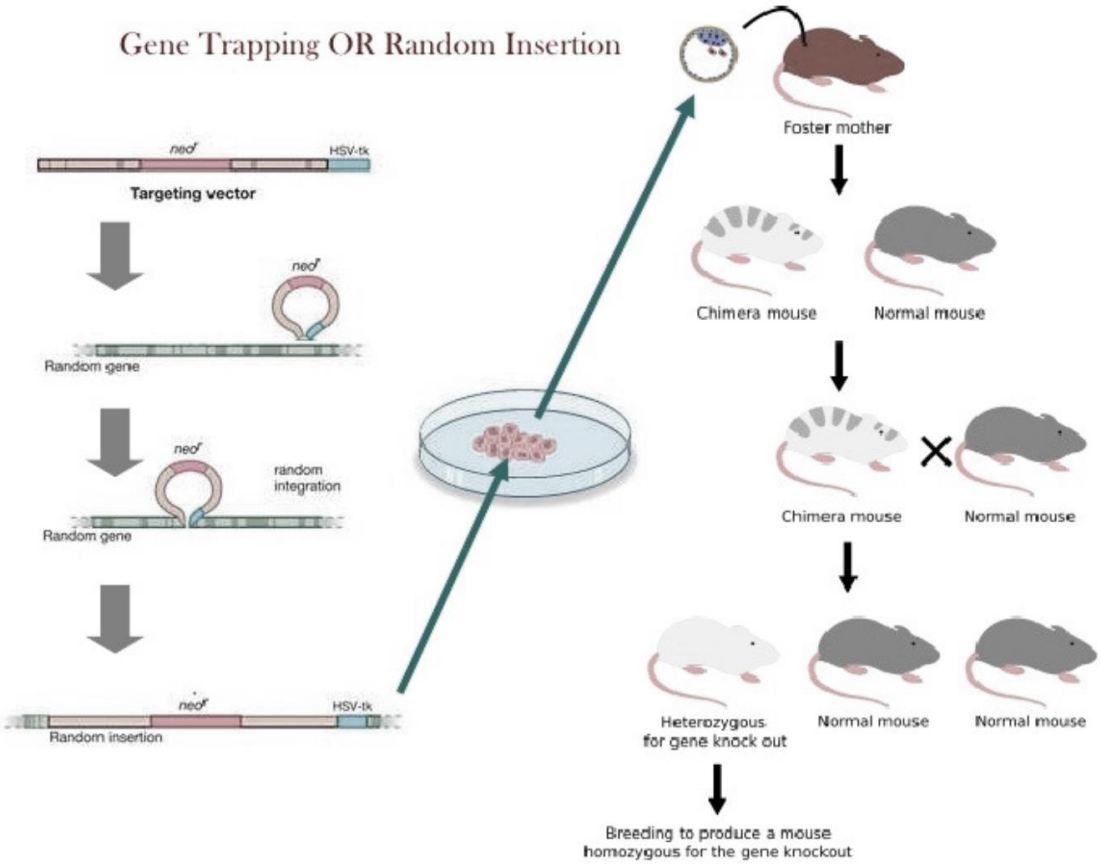
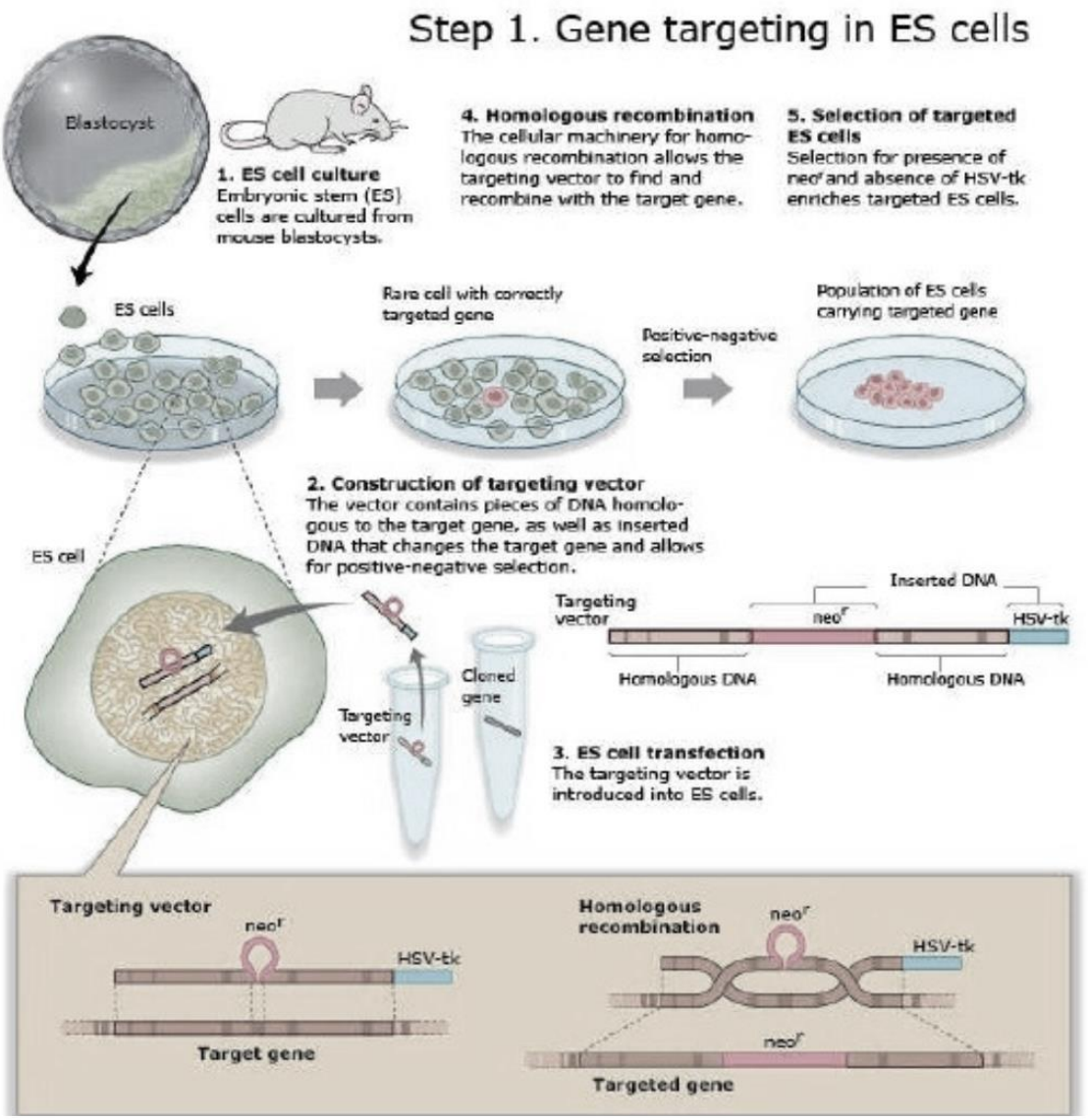
RANDOM GENE INSERTION

The classic method used for the generation of transgenic mice is called « pronuclear injection ». The transgene is injected into a fertilized mouse egg and then integrates at random positions in the genome.





TARGETED GENE INSERTION

The early vectors used for gene insertion could, and did, place the gene (from one to 200 copies of it) anywhere in the genome. However, if you know some of the DNA sequence flanking a particular gene, it is possible to design vectors that replace that gene. The replacement gene can be one that

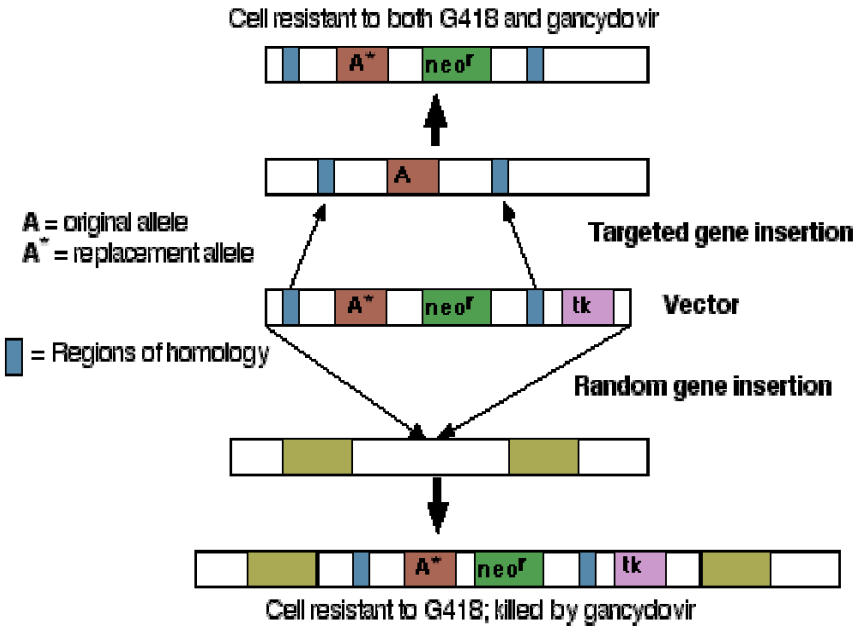
- restores function in a mutant animal or
- knocks out the function of a particular locus.

In either case, targeted gene insertion requires

- the desired gene

- ***neo^r***, a gene that encodes an enzyme that inactivates the antibiotic neomycin and its relatives, like the drug G418, which is lethal to mammalian cells;

- ***tk***, a gene that encodes **thymidine kinase**, an enzyme that phosphorylates the nucleoside analog **ganciclovir**. **DNA polymerase** fails to discriminate against the resulting nucleotide and inserts this nonfunctional nucleotide into freshly-replicating DNA. So ganciclovir kills cells that contain the *tk* gene.



Step 1: Treat culture of ES cells with preparation of vector DNA.

Results:

- **Most cells** fail to take up the vector; these cells will be killed if exposed to G418.

- In a **few cells**: the vector is inserted randomly in the genome. In random insertion, the entire vector, including the ***tk*** gene, is inserted into host DNA. These cells are resistant to G418 but killed by gancyclovir.

- In **still fewer cells**: homologous recombination occurs. Stretches of DNA sequence in the vector find the homologous sequences in the host genome, and the region between these homologous sequences replaces the equivalent region in the host DNA.

Step 2 : Culture the mixture of cells in medium containing both G418 and ganciclovir.

- The cells (the majority) that failed to take up the vector are killed by G418.
- The cells in which the vector was inserted randomly are killed by gancyclovir (because they contain the *tk* gene).
- This leaves a population of cells transformed by homologous recombination (enriched several thousand fold).
- **Step 3:**Inject these into the inner cell mass of mouse blastocysts.

Knockout Mice:

Step 1: Generating a targeting vector

The first step in making a knockout mouse is identifying the region of the gene that will be deleted. Because the entire mouse genome sequence is already known, it is relatively simple to look up the chromosomal location and nucleic acid sequence of the gene of interest. Once the segment of the gene that will be deleted has been mapped out, the nucleic acid sequences of the DNA segments that appear on the chromosome before and after that gene must also be identified.

Once these tasks are completed, a targeting vector specifically tailored to the gene of interest is made (Figure 1). A targeting vector is a long stretch of DNA made up of smaller pieces of DNA that have been joined together. Next, in order to make the targeting vector detectable, the scientists insert a marker gene into the middle of the vector; this marker is in some way able to "report" when it is present in a cell.

Currently, the neomycin-resistance gene, called *NeoR*, is a popular marker gene of choice for generating knockout mice. The antibiotic neomycin is toxic to mouse cells because they do not normally contain the *NeoR* gene. However, when the *NeoR* gene is added to mouse cells, these cells can survive in the presence of neomycin. Within the targeting vector, the *NeoR*gene is located between two other pieces of DNA: the "right arm" and the "left arm" of the targeting vector. The right arm of the targeting vector contains DNA with a nucleic acid sequence that matches the stretch of DNA immediately *before* the gene segment that will be deleted. The left arm of the targeting vector contains DNA with a nucleic acid sequence that matches the stretch of DNA immediately *after* the gene segment that will be deleted. The right and left arms of the

targeting vector facilitate homologous recombination between the targeting vector and the target gene, thereby enabling the *NeoR* gene to replace the target gene segment.

The targeting vector also contains one additional piece of DNA, called a **negative selection marker gene**. This gene is located at the right end of the targeting vector, after the right arm. The thymidine kinase (*TK*) gene from the herpes simplex virus is the most commonly used negative selection marker gene. Normally, mouse cells can grow in the presence of the antiviral drug ganciclovir. The *TK* gene is considered a "cell suicide gene," however, as cells containing the *TK* gene convert ganciclovir into a lethal toxin.

Why is it necessary to include a cell suicide gene as part of the targeting vector? The reason is purely a matter of identification - specifically, the *TK* gene helps researchers locate cells that have correctly replaced the targeted gene segment with the *NeoR* gene. Often, mouse cells randomly insert the targeting vector in the wrong chromosomal location. If random insertion occurs, both the *NeoR* gene and the *TK* gene are inserted into the genome. As a result, the cells are resistant to neomycin, but they die in the presence of ganciclovir. In comparison, when the targeted gene segment is correctly replaced, the *TK* gene is not inserted into the chromosome along with the *NeoR* gene, so the resultant cells are resistant to both neomycin and ganciclovir. Therefore, the presence of the *TK* gene in the targeting vector allows researchers to efficiently screen for mouse cells that have correctly replaced the targeted gene segment by growing these cells in the presence of both neomycin and ganciclovir.

Step 2: Inserting the target sequence and selecting cells with the insertion

After the targeting vector is made, it is used to knock out one copy of the target gene in mouse embryonic stem (ES) cells

It is certainly possible to use the targeting vector to knock out one of the two copies of the target gene in a standard somatic cell. However, unless that cell is an ES cell, the knockout mutation cannot be incorporated into a growing embryo. Therefore, it would not be possible to study the effects of the knockout mutation in a developing mouse.

What makes ES cells so special? Primarily, it is their ability to become any one of the different adult cell types. When injected into a mouse embryo, the ES cells themselves are capable of maturing into some of the tissues of the developing mouse.A technique called **electroporation** is used. When ES cells are electroporated, a brief pulse of an electrical field is applied to the outside of the cells, creating a momentary increase in plasma membrane permeability and allowing the uptake of foreign DNA into the ES cells.

After the ES cells have been electroporated, they are grown in the presence of neomycin to select for those particular cells that have taken up the targeting vector. Next, the neomycin-resistant cells are grown in the presence of ganciclovir to select for those that have inserted the targeting vector at the correct location within the mouse genome.

Step 3: Identifying ES cells with the correct gene knocked out

Additional experiments using standard molecular biology techniques help researchers determine whether the target gene has been fully knocked out in the ES cells that are resistant to both neomycin and ganciclovir. After these experiments are complete, only the ES cells that have had one copy of the target gene knocked out remain .These ES cells are heterozygous for the knockout mutation. Although these cells will grow and divide in culture, they cannot form an embryo that will develop into a mouse on their own.

Step 4: Injecting heterozygous knockout ES cells into a developing embryo and transferring the embryo into a mouse

The first step involves injection of these cells into a developing mouse embryo. This step allows the heterozygous knockout ES cells to become part of the developing embryo. Then, because they are ES cells, the heterozygous knockout cells are incorporated throughout the embryo and are capable of becoming any type of tissue within the developing mouse. Therefore, like a patchwork quilt, the developing embryo contains a mixture of its own original cells and the heterozygous knockout cells. Because of this cellular mixing, the resultant mouse is called a **chimeric mouse.**

Step 5: Mating chimeric mice to yield homozygous knockout mice

To produce a mouse that is homozygous for the target gene knockout, chimeric mice capable of passing the knockout mutation on to their offspring must be identified.

These chimeric mice can be identified by crossing them with normal white mice. If black offspring are produced from such a cross, a chimeric mouse is capable of passing the knockout mutation on to its offspring. When this is the case, 50% of the black offspring are heterozygous for the knockout mutation in all of their cells

Standard molecular biology techniques can be used to determine which of the black offspring are heterozygous for the knockout mutation. Then, to produce a homozygous knockout mouse, a heterozygous knockout male is mated to a heterozygous knockout female.Twenty-five percent of the resulting offspring will be homozygous knockout mice, which can, again, be readily identified using standard molecular biology techniques.

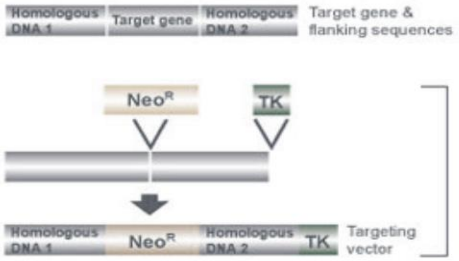
Step 6: Phenotypic characterization of homozygous knockout mice

After the homozygous knockout mice have been created, researches must then characterize the phenotypes associated with the loss of the target gene. In theory, every measurable phenotype must be examined in order to determine every possible function of the knocked-out gene. The measurement of a given phenotype in a knockout mouse would then be compared to measurements of the same phenotype in a **wild-type mouse** (a mouse that has not been genetically engineered for specific traits) in order to identify the functions that are altered in the knockout mouse.

Phenotypes such as size, weight, metabolism, behavior, bone development, neurological function, reproduction, and aging can be easily measured. If the knocked-out gene is required for development, however, it may not be possible to produce homozygous knockout mice. In this case, researchers may study heterozygous knockout mice, or they may instead turn to other types of knockout mice, such as **conditional knockout mice** (in which the target gene is inactivated in response to a specific stimulus) or **tissue-specific knockout mice** (in which the target gene is inactivated in only one or several tissues).

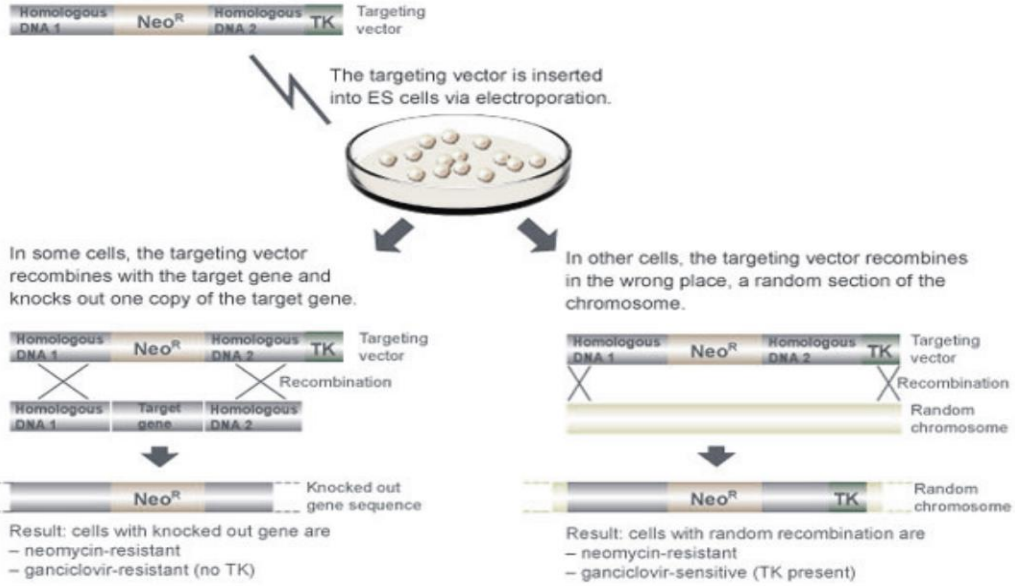
Making a knockout mouse

Step 1: Designing the targeting vector



The markers Neo^R and TK are inserted into the target gene sequence to make a targeting vector sequence.

Step 2: Inserting the targeting vector into ES cells



Step 3: Selecting cells

Only the cells that have successfully incorporated the targeting vector into the target gene survive in the presence of neomycin and ganciclovir (shown in red).

Step 4: Injecting cells into a new embryo

Cells containing the targeting vector are then selected and injected into a normal developing mouse embryo.

Step 5: Breeding

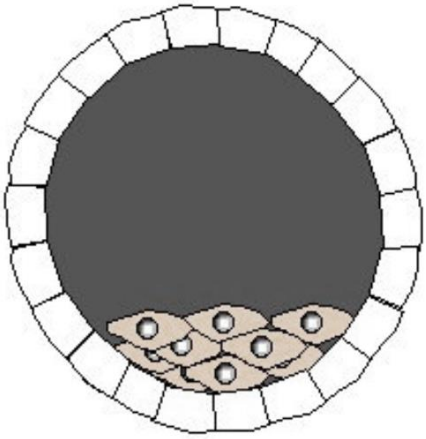
The resulting chimeric (spotted) mouse contains a mix of its own cells and the heterozygous knockout cells. This mouse is bred with a normal (white) mouse.

Among their offspring are mice that are capable of passing the knocked-out gene

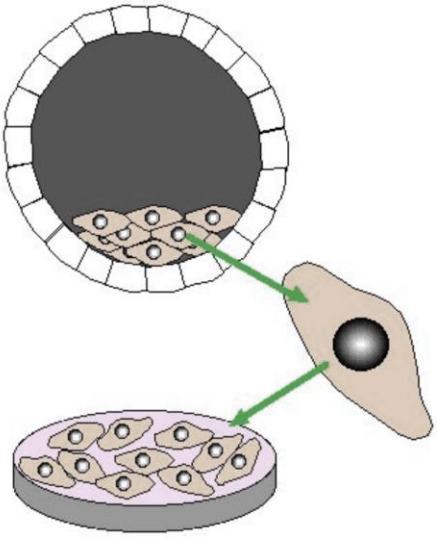
KNOCKOUT MOUSE

A **knockout mouse** has had both alleles of a particular gene replaced with an inactive allele. This is usually accomplished by using homologous recombination to replace one allele followed by two or more generations of selective breeding until an breeing pair are isolated that have both alleles of the targeted gene inactivaated or knocked out. Knock out mice allow investigators determine the role of a particular gene by observing the phenotype of individuals that lack the gene completely.

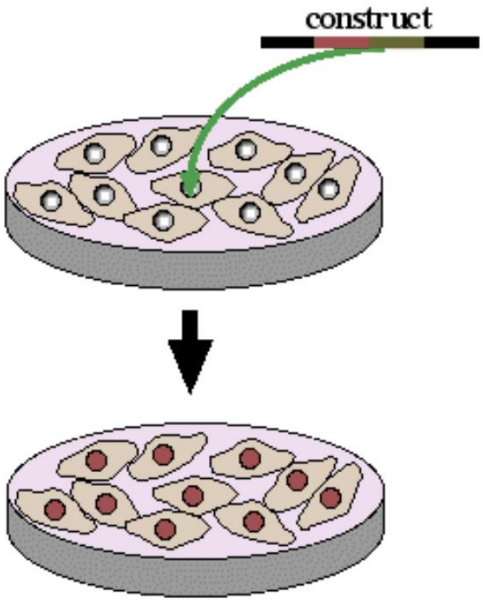
Step 1. Isolate developing embryo at blastocyst stage. This embryo is from a strain of mice with gray fur.



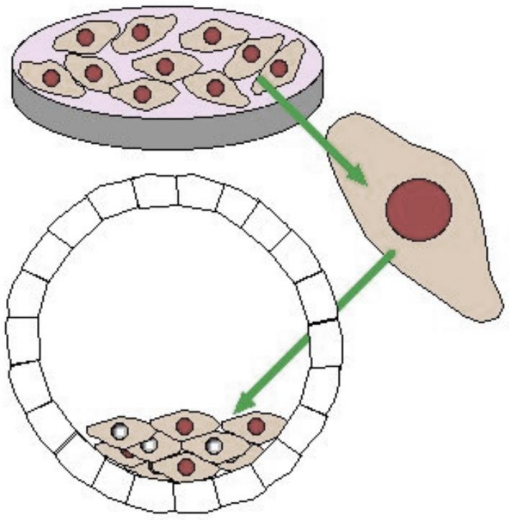
Step 2. Remove embryonic stem cells from gray-fur blastocyst. Grow stem cells in tissue culture.



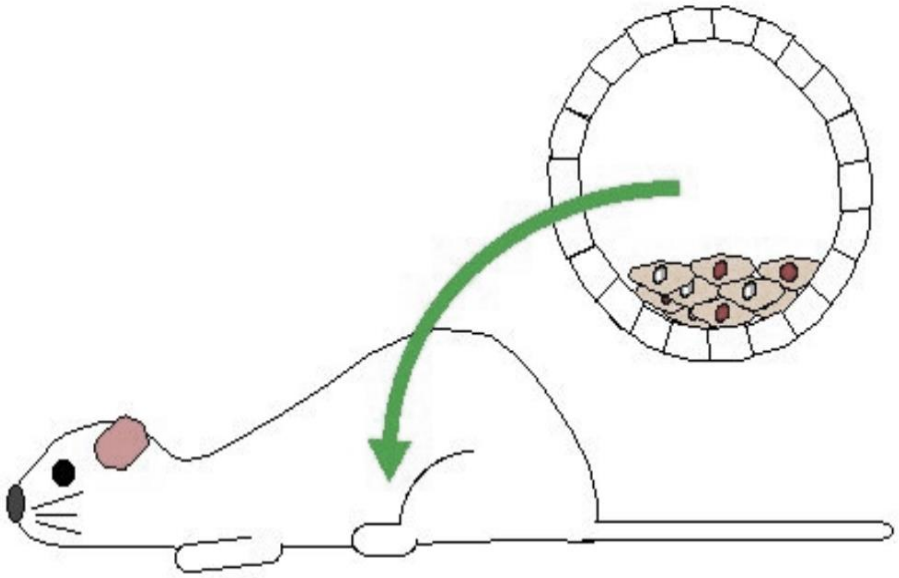
Step 3. Transfect stem cells with homologous recombination construct. Select for homologous recombination by growing stem cells in neomycin and gancyclvir.



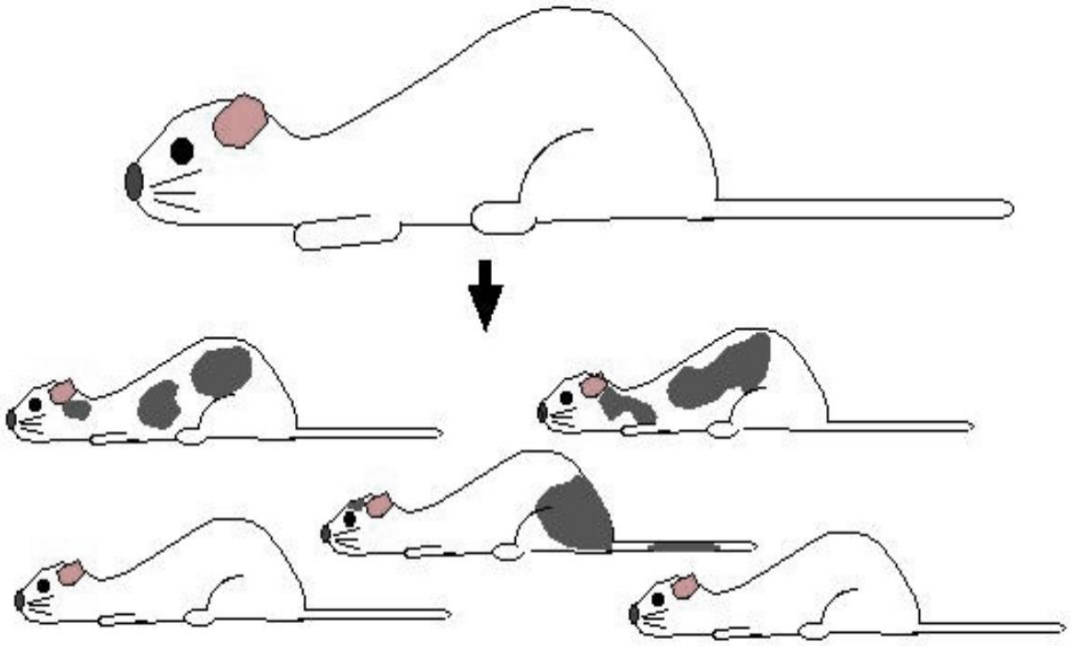
Step 4. Remove homologously recombined stem cells from petri dish and inject into a new blastocyst that would have only white fur.



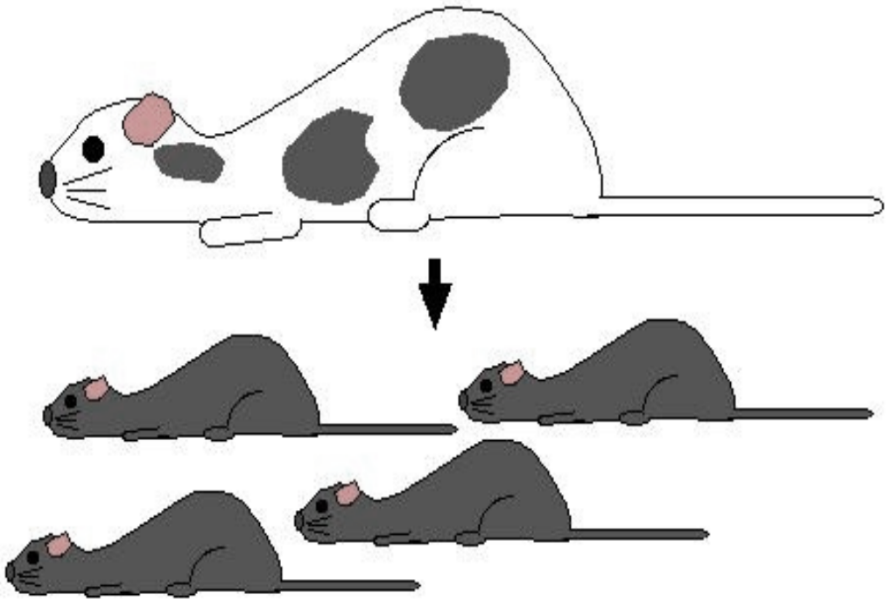
Step 5. Implant several chimeric blastocysts into pseudo-pregnant, white fur mouse.



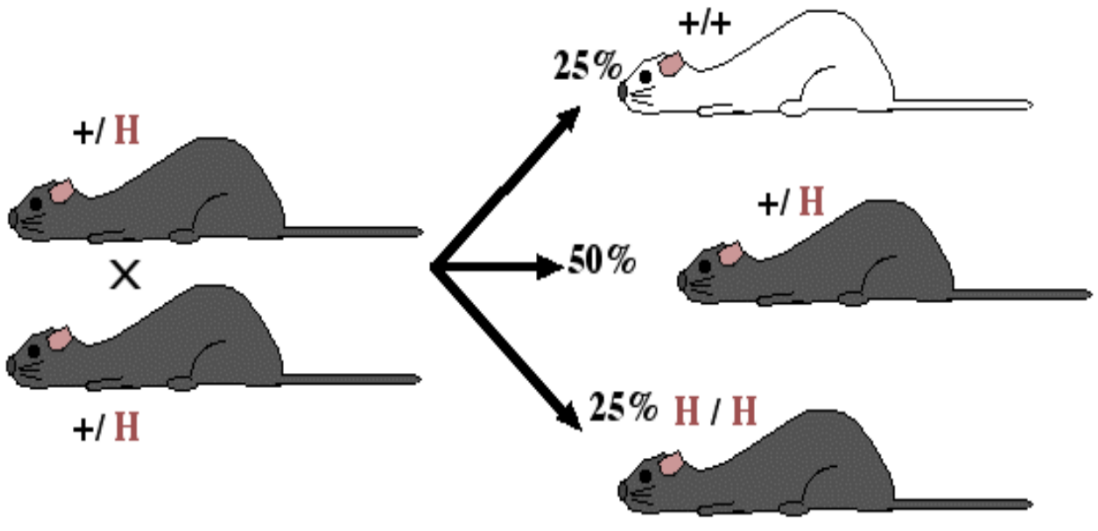
Step 6. Mother will give birth to a range of mice. Some will be normal white fur mice but others will be chimeric mice. Chimeric mice have many of their cells from the original white fur blastocyst but some of their cells will be derived from recombinant stem cells. Fur cells from recombinant stem cells produce gray patches which are easily detected.



Step 7. Mate the chimeric mice with wild-type white fur mice. If the gonads of the chimeric mice were derived from recombinant stem cells, all the offspring will have gray fur. Every cell in gray mice are heterozygous for the homologous recombination.



Step 8. Mate heterozygous gray mice (+/ H) and genotpye the gray offspring. Identify homozygous recombinants (H / H) and breed them to produce a strain of mice with both alleles knocked out. The pure breeding mouse strain is a "knockout mouse".



knockout mouse

KNOCK-IN MICE

The Cre/*loxP* system can also be used to

- remove DNA sequences that block gene transcription. The "target" gene can then be turned **on** in certain cells or at certain times as the experimenter wishes.
- replace one of the mouse's own genes with a new gene that the investigator wishes to study.

Such transgenic mice are called "knock-in" mice.

References and further reading

Tissue Engineering--Current Challenges and Expanding Opportunities by Linda G. Griffith and Gail Naughton (2002)

Textbook of Animal Biotechnology, by B Singh (Author), S K Gautam (Author), M S Chauhan (Author)

Animal Biotechnology (3rd Edition), by M.M. Ranga (Author)