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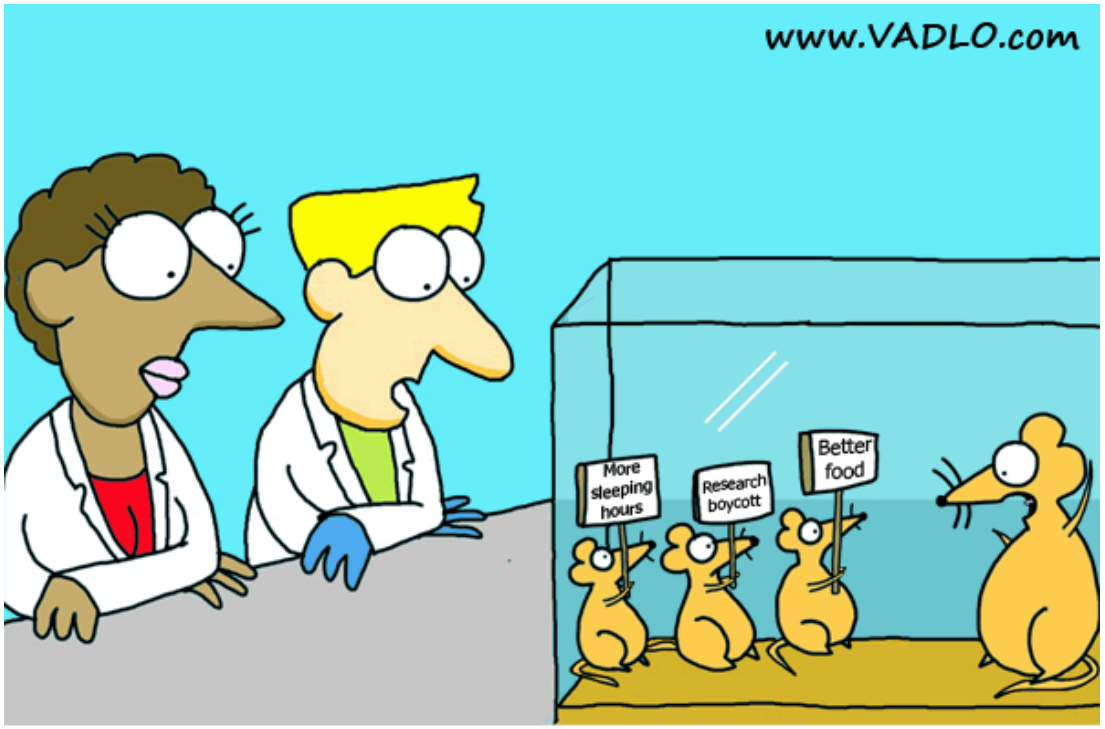
Applications of Transgenic Animals



"Our mistake.
We introduced a politician's genes in that one!"

Transgenic Animals

- Transgenic animals are created by **deliberately inserting a gene into the genome of an animal.**
- Recombinant DNA methodology is **used to construct the gene that is intended to express desirable qualities** during the growth and development of **the recipient animal.**
- This process is termed **'transgenesis'** which includes the **addition of foreign genetic information to animals and specific inhibition of endogenous gene expression.**
- Transgenic animal models allow unprecedented control over manipulation and visualization of genes and gene products.
- Due to their versatility, they have become a mainstay of the biomedical and basic science research landscape.
- Major advances and wide acceptance of the Cre/loxP technology has allowed detailed physiological and molecular genetic analysis of every conceivable system in biology.
- While most of these studies in mammalian systems have been largely utilized the mouse, it is anticipated that transgenic approaches will only become more prevalent in other species such as the rat and pig.

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Importance of Transgenic Animals

- Medical importance
  - Disease model
  - Bioreactors for pharmaceuticals
  - Xenotransplantation
- Agricultural importance
  - Disease resistant animals
  - For improving quality and quantity of milk, meat, eggs and wool production
- Industrial importance
  - Toxicity sensitive transgenic animals to test chemicals.
  - Spider silk in milk of goat

- *Transgenic disease models* (Models for understanding the human diseases e.g. AIDS mouse, Alzheimers mouse, Oncomouse)
- *Transpharmers* (Proteins obtained have medical and pharmaceutical importance)
- *Xenotransplanters (*organ transplant)
- *Transgenic food sources* (e.g. superpig, superfish)
- *Transgenic biological models* (Study gene expression and developmental processes)

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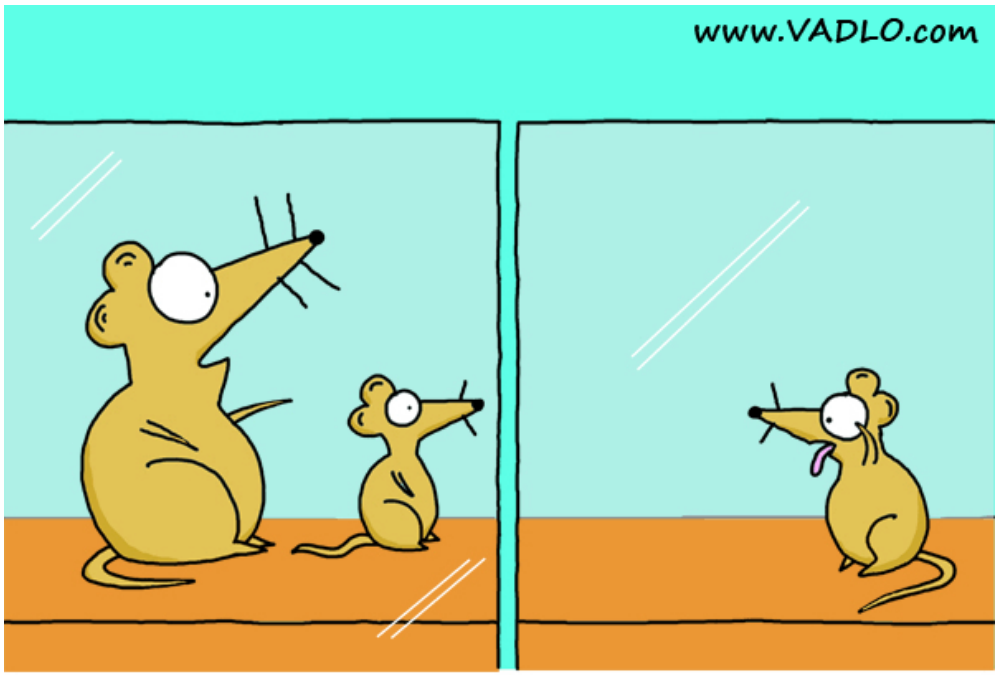
Knock Out Mice



"Don't play with him, he is **Wild Type**."

Gene knock out

- Gene knockout (KO) is the genetic technique comprised of the biotechnological tools which are used to **make the gene inoperative or inactivate in the whole body of the organism by permanently changing or mutating the gene of interest in the organism.**
- This approach can be **used to study either gain of function or loss of function phenotypes.**
- This can only be achieved at **embryonic stage**.
- **Homologous recombination** is used to swap over the normal copy of an exon with a mutated version of the DNA.
- The **genes can be made non functional by deleting or replacing or mutating** the coding sequences of selected genes.
- There is **no need to remove the complete gene** from the organism. The **mutated or altered gene sequences translate a truncated and non-functional protein.**
- As a result, the gene is no more functional and has been completely omitted from the organism.

Knock out mouse refers to the mouse in which one or more genes have been completely knocked out. The gene has to be completely inactivated from the whole body of the mouse. Therefore, the gene needs to be knocked out at embryonic stage in the embryonic stem (ES) cells.

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Significances of Knock Out Mice

- **Knockout mice** are used to study what happens in an organism when a particular gene is absent.
- Studying **knockout mice** can provide information about **how the knocked-out gene normally functions, including the gene's biochemical, developmental, physical, and behavioral roles.**
- The knockout mice for the mouse homologs of human **glucokinase and Apc genes** clearly indicated the direct **causality for diabetes and colon cancer, respectively.**

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Steps for the generation of KO mouse

1. Identification of gene of interest:

- The gene of interest need to be identified that needs to knock out.
- Once the gene selected for inactivation, the information of chromosomal locations, gene size and exon/ intron should be collected.
- The major restriction site to be identified and restriction map of the genomic locus to be prepared to plan better cloning strategies in the replacement vector.
- The flanking region should also be examined to avoid any disruption of neighboring genes during the recombination process.

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2. Construction of targeting vector:

- The **efficient targeting replacement vector** needs to be constructed **to introduce the targeted mutations or to replace the gene of interest in ES cells.**
- The drug resistance marker i.e. neomycin in the targeting replacement vector should be able to replace the essential coding region of the gene of interest in the genetic locus.
- The popular replacement vector possesses both positive and negative selection marker with two **homology arms flank with positive drug selection marker (neo^r) for homologous recombination which is placed adjacent to the targeting arms and negative selection marker (HSV-tk)** which confers sensitivity to gancyclovir, a cytotoxic nucleotide analog **(for screening the non homologous recombination) .**
- The DNA strands of the vector and the cells are exchanged through the process of homologous recombination. **At least 2 kb of sequence homology is needed on the arms of the targeting gene in the ES cell with arms of resistance gene in the vector for the recombination process to take place.**
- Further, the homology of 6-14 kb considered as typical for the recombination.
- Therefore, the targeting vector should be designed by incorporating this important feature of homologous recombination to boost the chance of success while developing a KO animal.
- The homologous recombination is the key feature for generating the knockout mouse while nonhomologous recombination for generation of transgenic animal.

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3. Isolation of embryonic stem (ES) cells:

- Since the purpose is to produce an animal in which gene has been knocked out from the complete body therefore we need to target the ES cells which form the complete body.
- Embryonic stem (ES) cells are pluripotent cell lines having the capacity of self-renewal and differentiation.
- The ES cells are harvested from the fertilized embryo at the balstocyst stage 4-5 days post-fertilization.
- The inner mass of blastocyst has mass of 10-20 cells and that inner mass recovered and culture in suitable culture media for expansion.
- The ES cells recovered from the blastocyst of the fertilized egg of a female mouse and that mouse is called as donor mouse.

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4. Electroporation of vector into the ES cells:

- Electroporation is extremely efficient approach for transfecting the foreign DNA into the cells with the use of electrical pulse that generate temporary pores in the cell membrane.
- An electrical pulse for a few microseconds to a millisecond is passed through the cell suspension to disturb the phospholipid bilayer and form the temporary pores in the membrane.
- The raised electric potential across the cell membrane permit the charged DNA to pass through the pores of the membrane into the cells where homologous recombination take place.

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5. Homologous Recombination in the cultured ES cells :

- Homologous recombination have important role in preserving and duplicating the genome by providing critical support for the replication of DNA and maintenance of telomere.
- The homologous recombination metabolizes DNA in all form of life and provides template dependent repair of DNA gaps, double stranded breaks and cross-linking of strand exchanged.
- Taking advantage of these properties of homologous recombination process, the scientist implies homologous recombination for exchanging of DNA strands between targeted replacement vector and gene of interest at designated genetic locus to inactivate the gene of interest.
- The double-strand break repair (DSBR)/ Holliday junction model and synthesis-dependent strand annealing (SDSA) are the two important models for homologous recombination.

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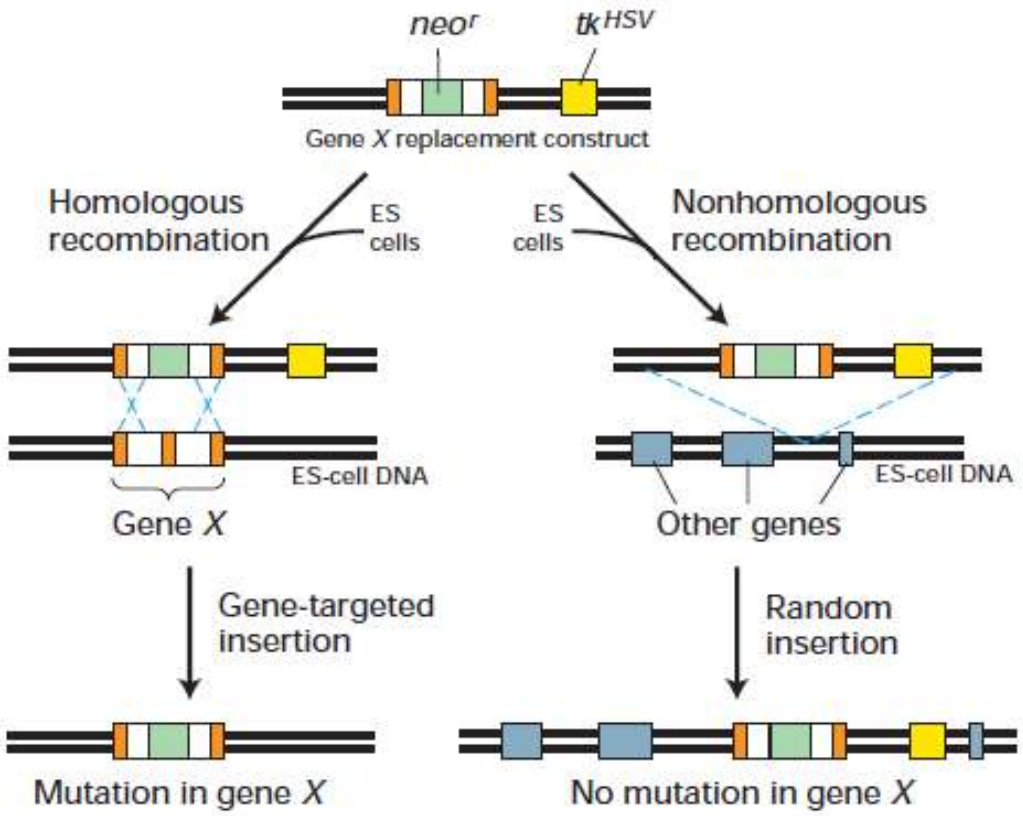
6. Selection of ES cells with resistance marker:

- The targeted events by **homologous recombination take place in very few cells or small percentage of cells** and there is need to select the cells in which homologous recombination took place.
- This can be done through some resistance marker and the **neomycin is the most common resistance marker.**
- The construct often is engineered having **target-gene sequence disrupted by the neo^R gene and the tk^{HSV} gene at one end, beyond the sequence of the target gene.**
- If **homologous recombination occurs only the target gene and neo^R gene will be inserted**.
- In the case of **non-homologous recombination all the three genes will be inserted (target gene, neo^R gene, tk^{HSV} gene).**
- On culturing the ES cells in the presence of the antibiotic, the cells who does not possess the property of antibiotic resistance dies and only the cells having antibiotic resistance gene can survive.
- Such survived cells allowed to expand in the culture.



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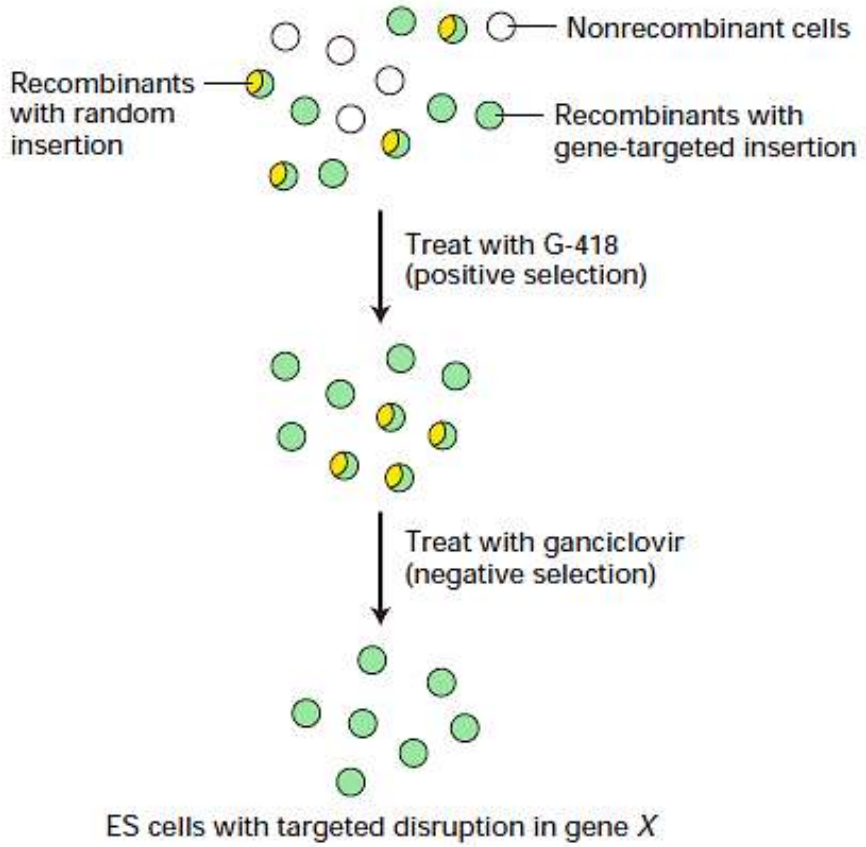
- A two-step selection strategy is used to select the ES cells that have undergone homologous recombination and as a result target gene is replaced by disrupted gene.
- **First,** the **ES cells are treated with G-418, neo^r which confers G-418 resistance to select only the recombinant ES cells as they have neo^r gene** and **secondly,** the **recombinant ES cells are treated with gancyclovir to select homologous ES recombinant cells because these cells do not have tk^{HSV} gene and non-homologous recombinant ES cells die because of presence of tk^{HSV} gene** (The viral thymidine kinase can convert the nucleotide analog ganciclovir into the monophosphate form; this is then modified to the triphosphate form, which inhibits cellular DNA replication in ES cells).
- Such survived cells allowed to expand in the culture.



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7. Transfer of selected ES cells to the mouse:

- The **selected ES cells transferred/ injected to the host embryo of recipient/surrogate mouse** for the development of pups.
- The **pups developed are chimeras** where gene of interest has been knocked out in some cells but some of the cells still having the gene.
- These **chimeras need to be screen and confirmed before moving further.**
- **If the recipient embryo and the ES cells are from two different strains of mice with different skin** will allows to **visually selecting the chimeras just by color.**

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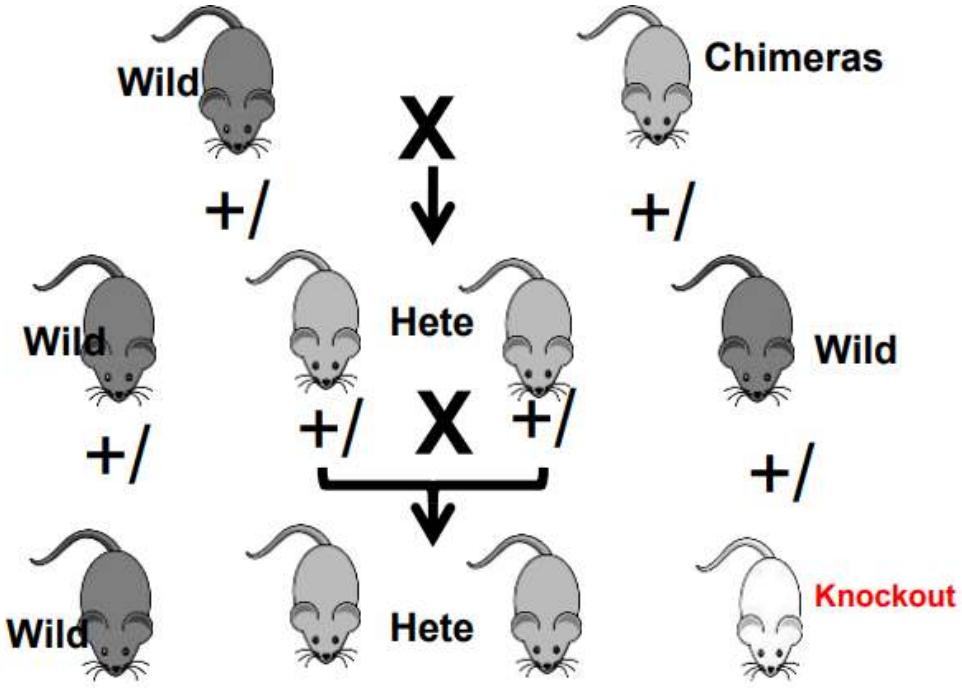
8. Mating to generate homozygous gene knockout mouse:

- The chimeric mouse mated with wild type mouse to incorporate the mutations in the germ line likely to produce 50 % heterozygous mouse.
- The heterozygous mouse carries one copy without gene (-) and one copy with (+) gene.
- The heterozygous mouse on mating with other heterozygous mouse produce 25% of the pups in which the gene of interest has been completely knocked out or loss the function of the gene.



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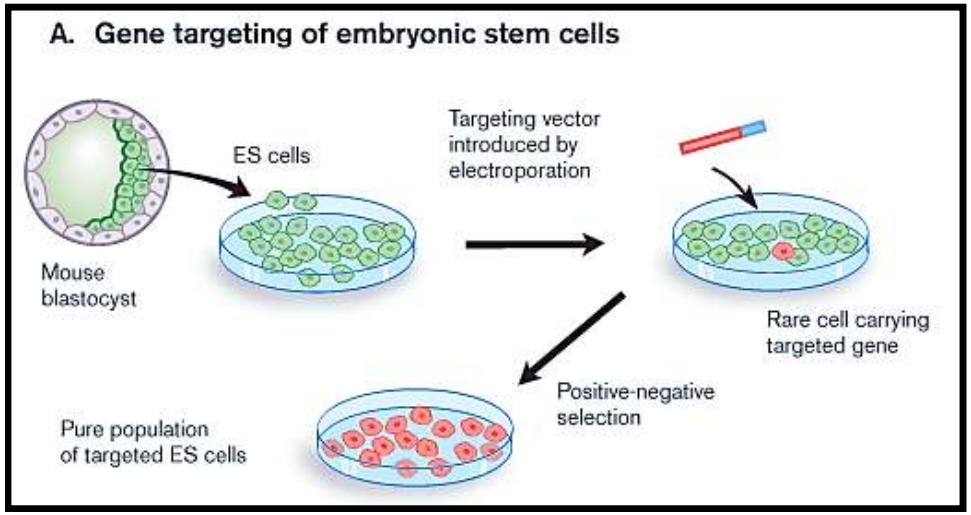
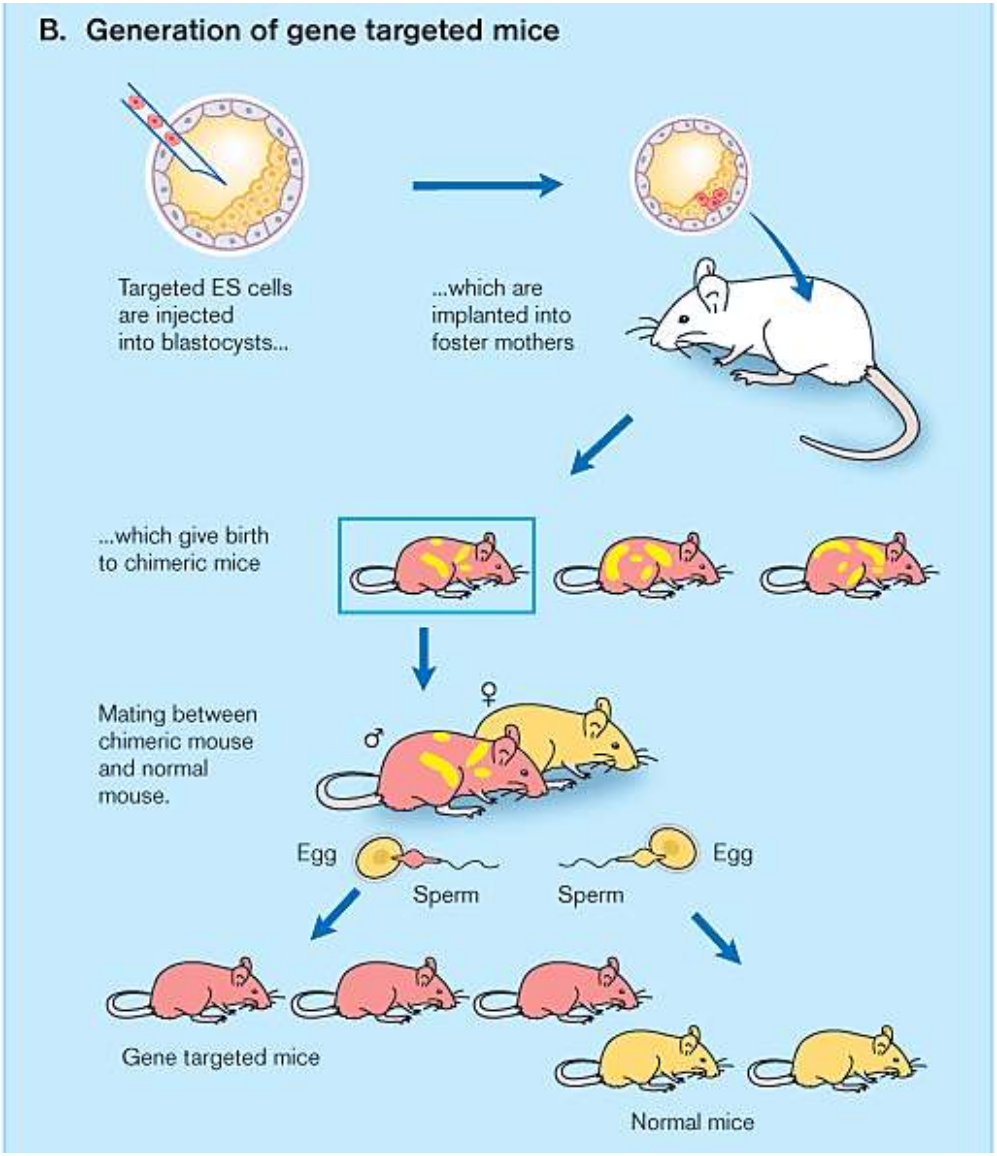
Procedure of knock out





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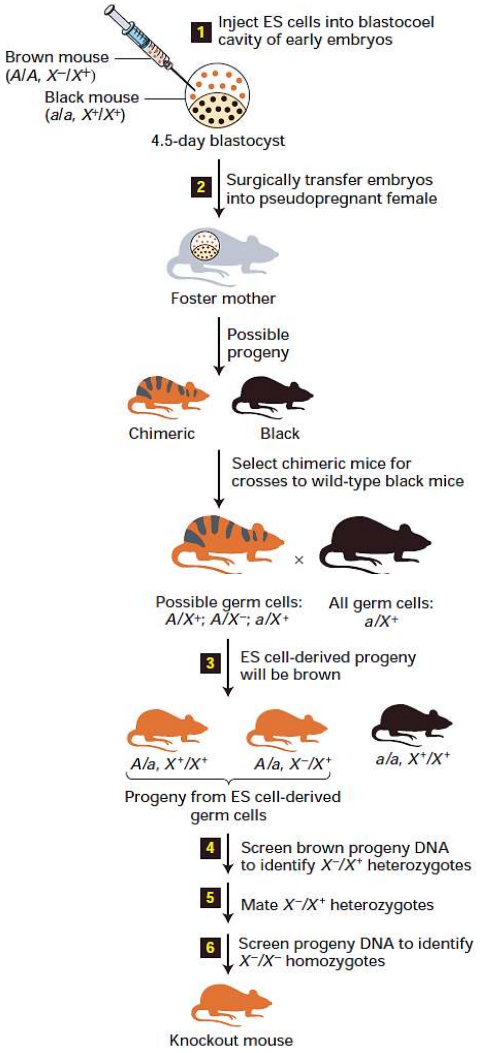
At a glance..



Step 1 : Embryonic stem (ES) cells heterozygous for a knockout mutation in a gene of interest (X) and homozygous for a dominant allele of a marker gene (here, brown coat color, A) are transplanted into the blastocoel cavity of 4.5-day embryos that are homozygous for a recessive allele of the marker (here, black coat color, a).

Step 2 : The early embryos then are implanted into a pseudo pregnant female. Those progeny containing ES-derived cells are chimeras, indicated by their mixed black and brown coats.

Step 3 : Chimeric mice then are backcrossed to black mice; brown progeny from this mating have ES-derived cells in their germ line.

Steps 4-6: Analysis of DNA isolated from a small amount of tail tissue can identify brown mice heterozygous for the knockout allele. Intercrossing of these mice produces some individuals homozygous for the disrupted allele, that is, knockout mice.

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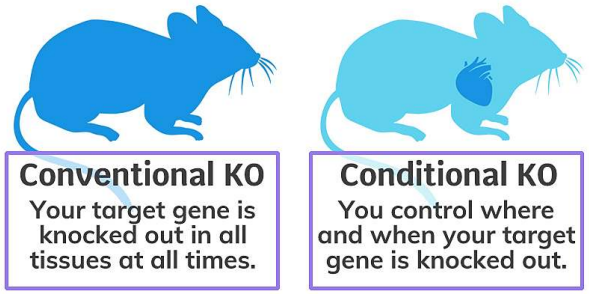
Development of conditional knockout mouse

- The constitutive KO of gene in mouse may sometime produce **undesirable result or may be embryonic lethal.**

- Therefore, there is need of **conditional KO model** where the conditional gene knockout enables the inactivation of a gene of interest in a **specific tissue and/or at a specific time point** while the other tissues of the mouse should retain its wild type function.



Constitutive, tissue specific and inducible knockout

- **(A)** In a wild-type mouse, the **GOI (GENE OF INTEREST)** is functional. In a **constitutive knockout** mouse the **GOI is not functional and therefore not expressed.** The generated Tg-mouse might show phenotypic changes, depending on which gene has been knocked out.
- **(B)** In a **tissue specific knockout mouse** the **GOI is not functional in a specific tissue, while normal gene functionality is detected in the rest of the organism**. In the figure the red targeted area illustrates that the gene is inactivated only in tendon tissues such as in the tail and Achilles tendons.
- **(C) Inducible knockout** systems allow the **inactivation of a GOI by the addition of an inducer (in defined doses) at a given time point and within a specific tissue, before that, gene of interest is functional.**

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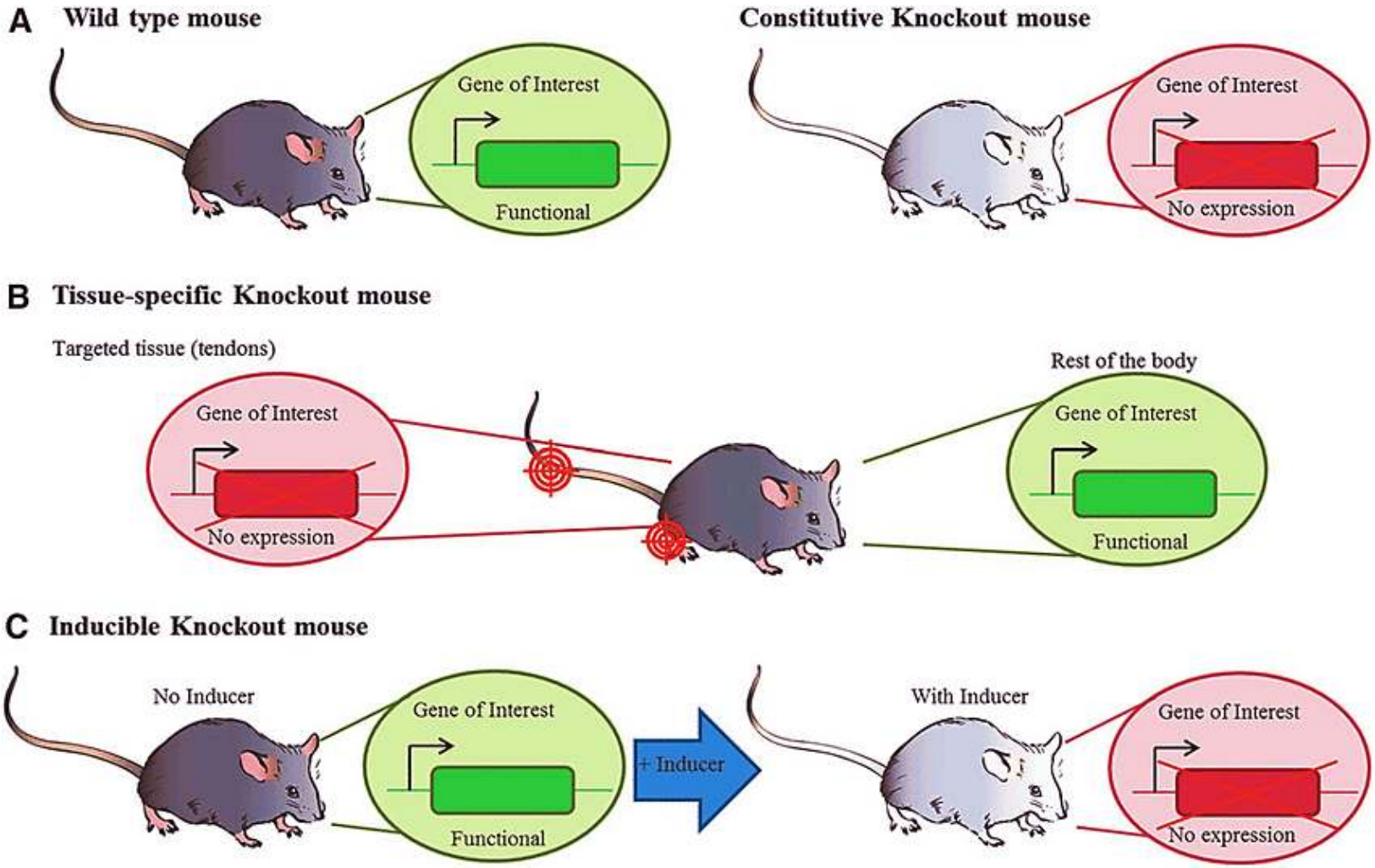
Constitutive, tissue specific and inducible knockout mouse models



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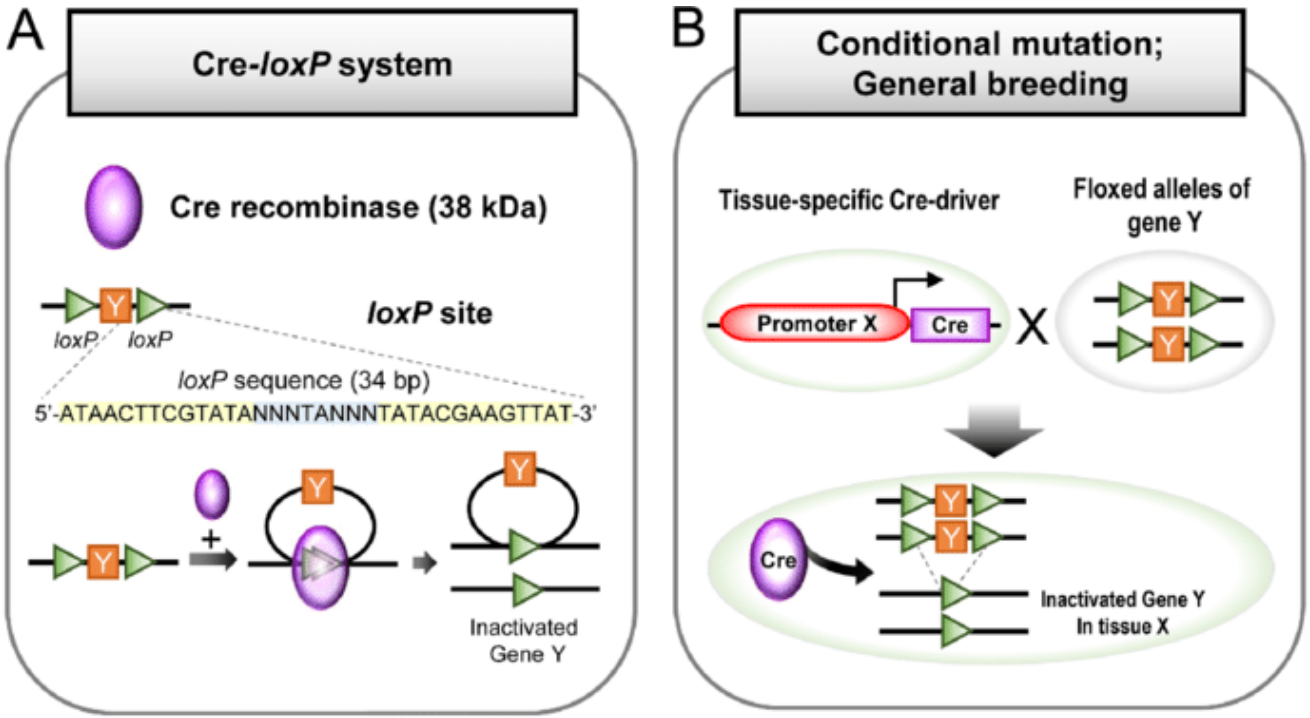
Mechanism of Cre-loxP system



(A) An overview of Cre-loxP system. 38 kDa Cre recombinase recognizes the loxP sites of specific 34 bp DNA sequences.

(B) General breeding strategy for conditional mutation using loxP and Cre driving mouse line. In principle, one mouse must have **a tissue-specific driven cre gene and another mouse have loxP flanked (floxed) alleles of interest gene Y.** Expression of Cre recombinase excises floxed loci and inactivates the gene Y.

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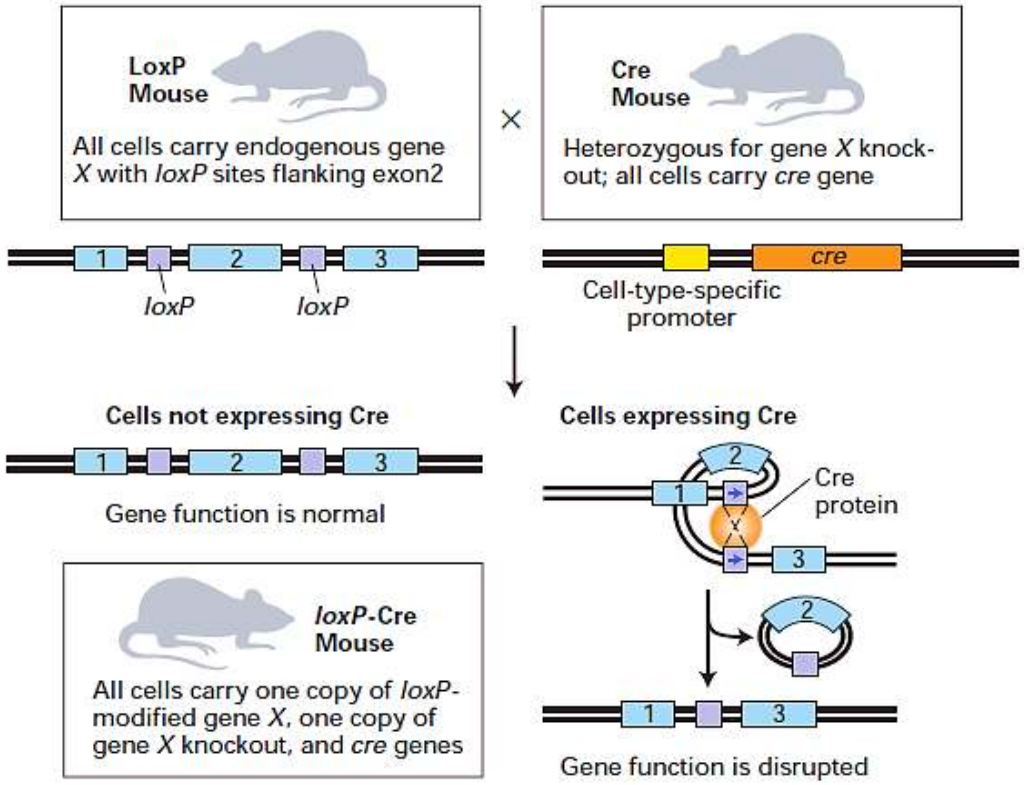
The loxP-Cre recombination system can knock out genes in specific cell types

- **Two loxP sites are inserted on each side of an essential exon (2) of the target gene X (blue)** by **homologous recombination,** producing a loxP mouse. Since the loxP sites are in introns, they do not disrupt the function of X.
- The **Cre mouse** carries **one gene X knockout allele and an introduced cre gene (orange) from bacteriophage P1** linked to a cell-type-specific promoter (yellow).
- The **cre gene is incorporated** into the mouse genome by **nonhomologous recombination** and does not affect the function of other genes.
- In the ***loxP*-Cre mice that result from crossing, Cre protein is produced only in those cells in which the promoter is active.**
- Thus **these are the only cells in which recombination between the *loxP* sites catalyzed by Cre occurs, leading to deletion of exon 2.**
- Since **the other allele is a constitutive gene *X* knockout, deletion between the *loxP* sites results in complete loss of function of gene *X* in all cells expressing Cre.**
- By using different promoters, researchers can study the effects of knocking out gene *X* in various types of cells.



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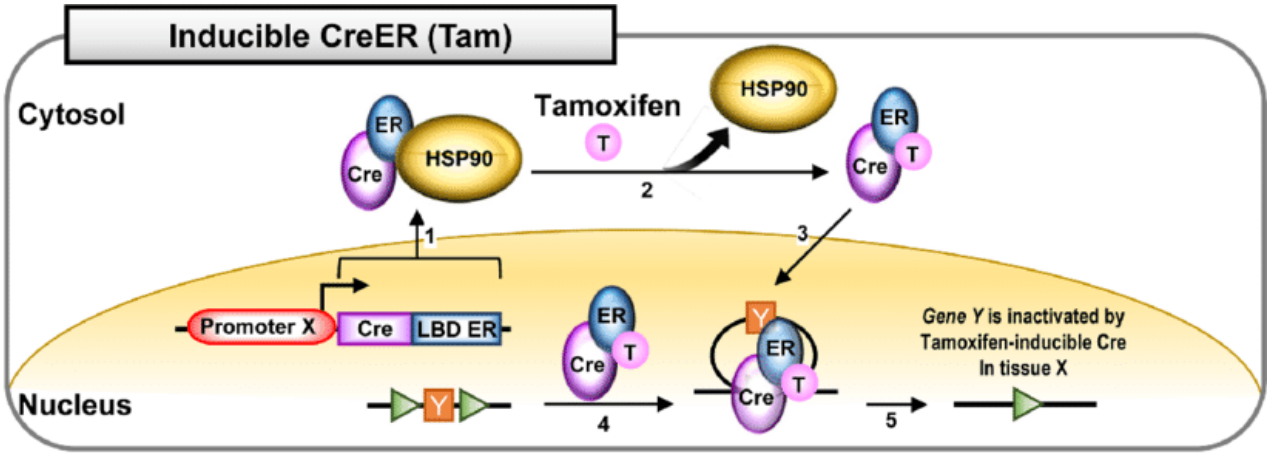
Mechanism of inducible knockout

- To be specific, the induction is performed based on the existence of **estrogen receptor (ER), a receptor that translocate from the cytoplasm to the cell nucleus to regulate gene expression after binding to the corresponding ligand, estrogen or tamoxifen.**
- To eliminate the effect of endogenous estrogen, the **binding domain of ER is artificially mutated and therefore it is only capable of binding to exogenous estrogen analogues, tamoxifen.**
- In this strategy, **Cre is ligated to the ER and this fusion protein (e.g., CreERT) will stay in the cytoplasm before the administration of tamoxifen**, leading to functional expression of the endogenous gene.
- However, **when tamoxifen is applied to the animal, either topically or through injection, it will bind to the ER as a ligand and thus activate the Cre-mediated recombination process.**
- This way, the knock-out model can be obtained and timing control of inactivation can be guaranteed.



Tamoxifen (Tam)-inducible System of estrogen receptor fused to Cre (CreER). In the absence of tamoxifen, expressed fusion protein, CreER, interacts with heat shock protein 90 (HSP90) and exists in cytoplasm (1). Administration of Tam disrupts the interaction of HSP90 with CreER (2). Interaction of ER with Tam induces nuclear translocation of Cre (3). In the nucleus, the CreER recognizes the loxP sites (4) and inactivates the gene Y in tissue X (5).

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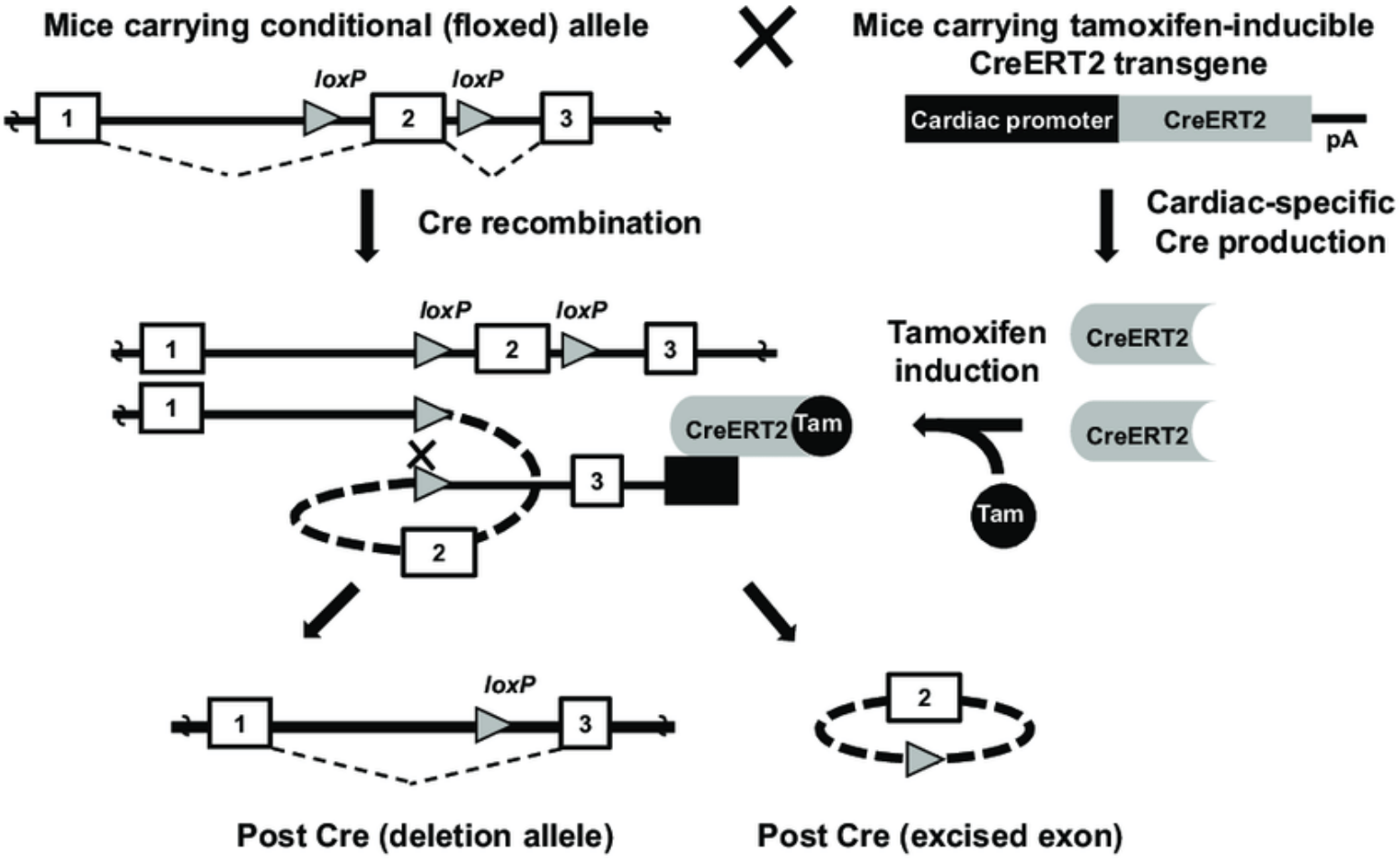
In details….



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Production of Pharmaceuticals

Pharmaceutical proteins

- Proteins are involved in a majority of the biochemical events that take place in all living organisms. Protein synthesis is directed by genes. All genes contain two major DNA regions. The region containing the genetic message proper (the 'coding region') is preceded by a regulatory region ('the promoter'), which determines when and in which organs a given gene must produce the corresponding protein.
- The techniques of genetic engineering allow the **association of the coding region from one gene with the regulatory region from another gene.**
- **The expression of these recombinant genes may be achieved in cultured cells, in transgenic animals or in plants. This leads to the production of the corresponding proteins, including pharmaceutical proteins.**

- **For Example:** Proteins started being used as pharmaceuticals in the 1920s with insulin extracted from pig pancreas. In the early 1980s, human insulin was prepared in recombinant bacteria and it is now used by all patients suffering from diabetes.

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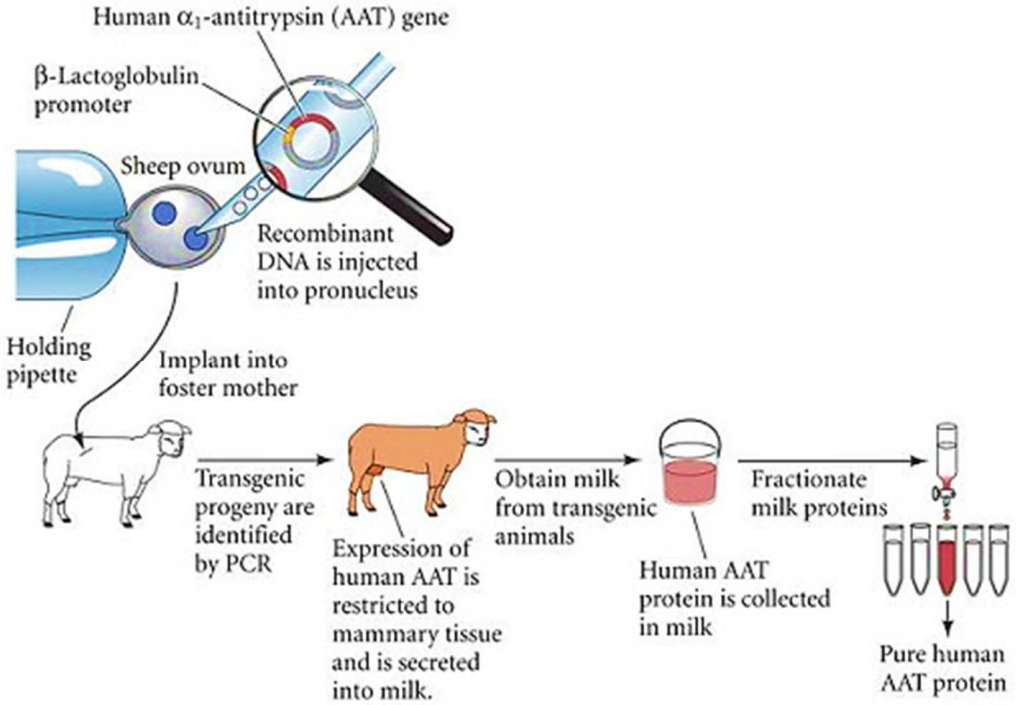
Transpharmers

- **Transgenic animals used for pharmaceutical production** are called **Transpharmers** which produces (over expression) **important human proteins in their saliva, milk, blood, urine and eggs after insertion of human genes.**
- **Sheep, cow, goats** are the main targets and the animals can be modified to produce the **protein of interest in their milk** to avoid animal sacrifice when obtaining the drug .
- Proteins **can be purified to produce medicines or hormones** to treat humans and can possibly be administered as medicinal milk itself.



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Transgenic animals are made by various process

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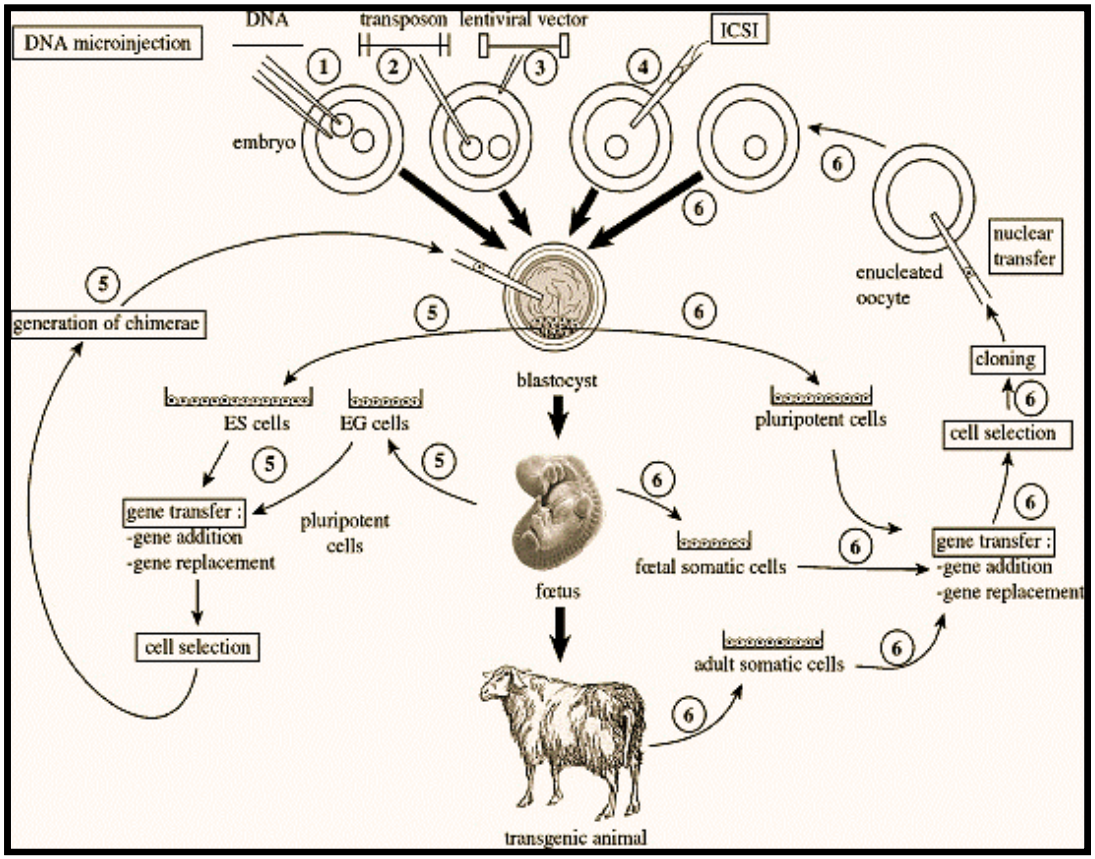

(1) DNA transfer via direct microinjection into a pronucleus or cytoplasm of embryo;

(2) DNA transfer via a transposon: the gene of interest is introduced in the transposon which is injected into a pronucleus;

(3) DNA transfer via a lentiviral vector: the gene of interest is inserted into a lentiviral vector which is injected between zona pellucida and membrane of oocyte or embryo.

(4) DNA transfer via sperm: sperm is incubated with the foreign gene and injected into oocyte cytoplasm for fertilization by ICSI (Intracytoplasmic Sperm Injection)

(5) DNA transfer via pluripotent cells: DNA is introduced into pluripotent cell lines (ES: embryonic stem cells: lines established from early embryo, EG: embryonic germ cells: lines established from the primordial germ cells of foetal gonads). The pluripotent cells containing DNA are injected into early embryos to generate chimeric animals harbouring the foreign gene.

(6) DNA transfer via cloning: the foreign gene is introduced into somatic cells, the nucleus of which are introduced into the cytoplasm of enucleated oocytes to generate transgenic clones.

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- Methods 4, 5 and 6 allow random gene addition and targeted gene integration via homologous recombination for gene addition or gene replacement including gene knock out and knock in.
- The genes to be transferred are known as **TRANSGENE.** Transgene must ideally contain:

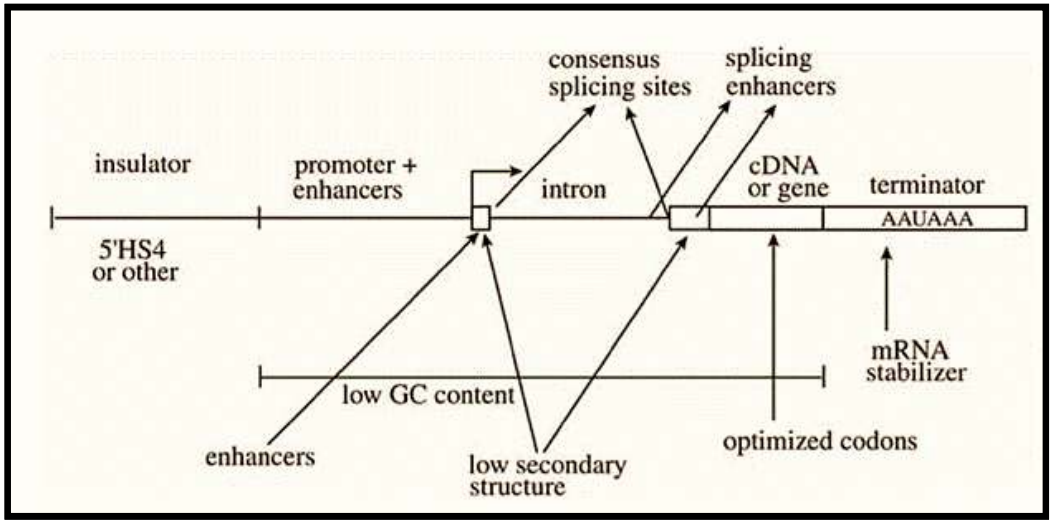
i. a promoter

ii. enhancers

iii. insulators

iv. introns and a

v. transcription terminator



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The different production systems

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- **Bacteria:** Some proteins form aggregates from which it is difficult to isolate the proteins of interest without denaturating them. Bacteria cannot always fold proteins in an appropriate manner and assemble subunits to form biologically active molecules.
- **Yeast** : Though they are unable to fold and secrete monoclonal antibodies in an appropriate manner, genetically modified yeast can secrete substantial amounts of recombinant proteins having carbohydrates almost similar to those found in human proteins. For example, recombinant hepatitis B vaccine prepared from yeast does not contain the disulphide bridges which have to be created in vitro.
- **Insect cells + baculovirus**: Insect cells do not add to proteins all the sugars which are present in human proteins. Recombinant proteins can be obtained from the following model
  - **silk gland**
  - **insect larvae haemolymph**
- **Animal cells (CHO cells)**: the proteins secreted in the culture medium are post-translationally modified essentially as their native counterparts.
- **Transgenic plants:** Foreign proteins may be stored in leaves, in seeds or both, according to the promoter used. Leaves are very abundant but it may be difficult to purify the protein of interest from them due to the presence of proteases or substances like polyphenols
- **Transgenic animals**: The advantages are the high and low cost production as well as the high quality of the proteins. A drawback may be the difficulty to separate the human proteins from their animal counterpart. Recombinant proteins can be obtained from the following animal products:
  - **Milk: It is presently the most mature system to produce recombinant proteins from transgenic organisms.**
  - **Blood, egg white , seminal plasma , urine .**

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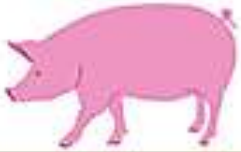
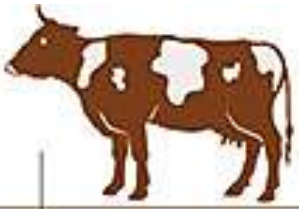
Some examples of pharmaceutical proteins



Protein	Human antithrombin (ATryn)	Human butyrylcholinester-ase (Protexia)	Human coagulation factor VIII fusion protein (Elocate)	Human fibrinogen (rhFIB)	Human C1 inhibitor (esterase inhibitor) (Ruconest)	Human myelin basic protein
Therapeutic use	Acts as anti-blood-clotting agent for young adults who are at risk of blood clots during pregnancy, surgery, or prolonged bed rest	Breaks down toxic nerve agents such as sarin gas into inactive compounds and then removes them from bloodstream	Stops bleeding in patients with hemophilia A, a blood disorder characterized by prolonged bleeding	Helps coagulate blood and treats afibrinogenemia, a condition in which a lack of fibrinogen leads to uncontrolled bleeding	Treats hereditary angioedema, which is swelling under the skin caused by allergic reactions in the body	Alleviates symptoms of multiple sclerosis
Company	rEVO Biologics, U.S.	PharmAthene, U.S.	Biogen, U.S.	Pharming, Netherlands	Pharming, Netherlands	AgResearch, New Zealand
Status	Approved as drug in the U.S. in 2009	Phase I clinical trial completed	In Phase III clinical trial	In Phase III clinical trial	Approved as drug in the U.S., Israel, and 28 European Union countries	In research and development[3]

MODEL-1 Mammary Gland, the Best Bioreactor Available

- The **mammary gland** has generally been considered the organ of choice to express valuable recombinant proteins in transgenic animal bioreactors because **milk is easily collected in large volumes**. Milk is currently the **best available bioreactor. Foreign proteins** are commonly reported to be produced **in transgenic milk at rates of several grams per liter.**
- Various **genetic modification is followed to alter the composition of milk.** The following table gives some important example of GM cattles.

Species	Category	Transgene	Origin	Effect/Goal
Cattle	Livestock	Lysozyme	Human	Milk composition
	Bioreactor	Lactoferrin	Human	Prophylactic treatment

There is no doubt that breast milk provides an ideal source of nutrition for infants, as well as promoting rapid growth and conveys more advantages than formula, as proteins in breast milk not only provide a well-balanced source of amino acids for nutritional needs, but also simultaneously aids in the defense against infections, enhances immune function, and promotes development of gut function.

To improve the nutritional content of infant formulas, attempts have been made to add human proteins,

- such as α-lactalbumin,
- lactoferrin, and
- lysozyme, to bovine milk by producing genetically modifying dairy cows and
- to remove the major allergenic bovine protein β-lactoglobulin

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Example 1: Mammary gland bioreactor system expressing recombinant human lysozyme (rhLZ)

- Currently Lu et al., (2016) introduced Large-scale production of functional **human lysozyme from marker-free transgenic cloned cows.**
- **Lysozyme is known to exert a wide range of antimicrobial activities** against pathogenic bacteria and viruses, and has been widely **used in food, pharmaceuticals, and clinical treatments, such as oral health care and antibiotic therapy.**
- Most lysozyme for pharmaceutical **use is purified from egg whites and thus may induce egg allergy among those sensitive to lysozyme.**
- Considering **pharmaceutical security and market demand**, large amounts of recombinant **human lysozyme (rhLZ)** should be produced.
- An alternative means of producing rhLZ is **through a mammary gland bioreactor system in transgenic farm animals expressing rhLZ in the mammary gland.**

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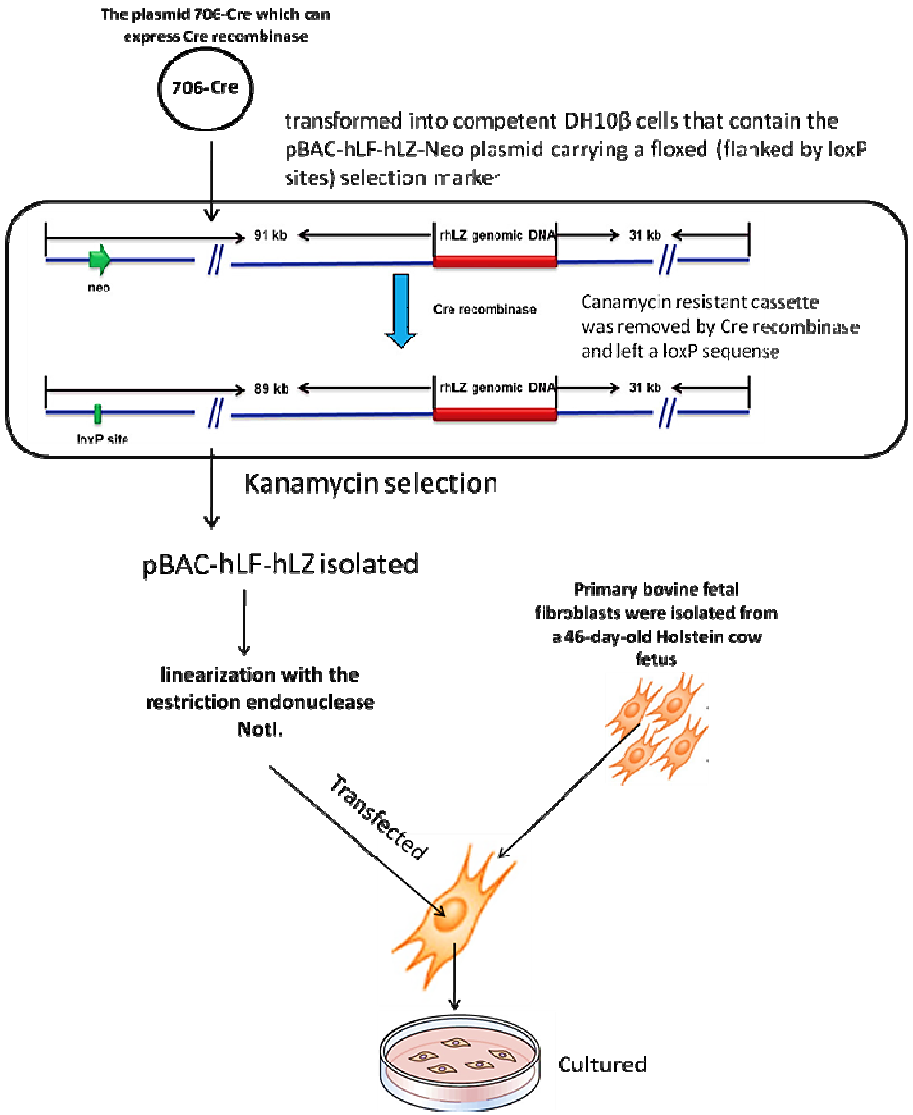
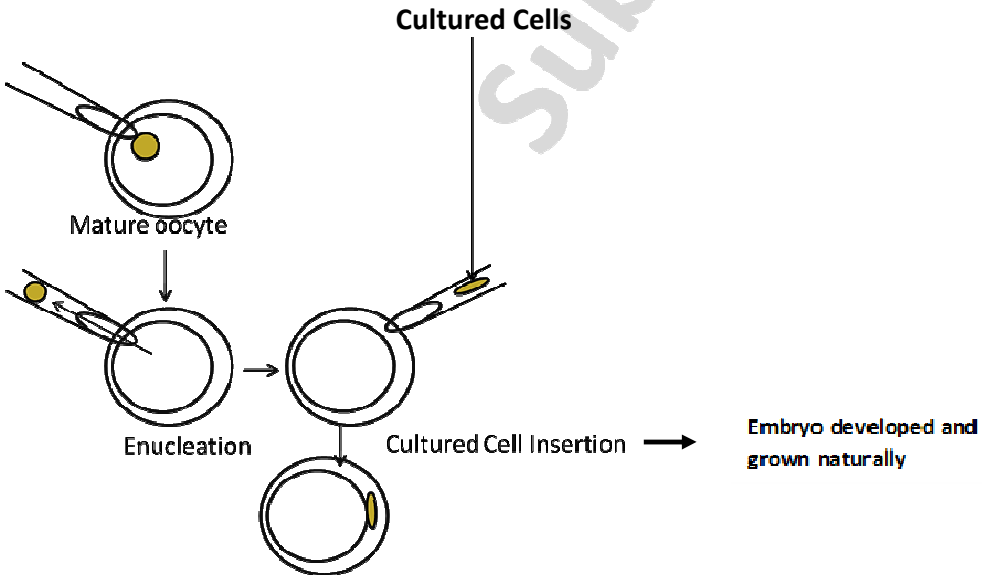
Procedure



DH10β cells

- The integration of the human lysozyme gene into the genome of the transgenic calves was confirmed by PCR and Southern blot analysis.
- The milk samples were purified using Sepharose column after removal of fat and casein.
- The rhLZ concentration in milk was as high as 3.1 g/L, which is sufficient to meet the comercial demand.
- The physicochemical properties of rhLZ was similar to those of naturally occurring hLZ.



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Model 2: Blood and Egg, Alternative Recombinant Protein Secretion Medium

- Animal blood, which collects secretions from many tissues, may be used as a source of recombinant proteins.
- **Human α-AT** was obtained at a high level from the **serum of transgenic rabbits.**
- **Human hemoglobin** has been produced in a **transgenic swine circulatory system.**
- **Transgenic chicken** stably produced a **human erythropoietin fusion protein** not only in their **serum and egg white** but also in the **egg yolk.**

Model 3: Urine- or Seminal-Fluid-Specific Expression Systems

- Urine is an abundant biological fluid already used to prepare proteins such as **gonadotropins for pharmaceutical use.**
- **Human growth hormone** gene driven by the **mouse uroplakin II gene promoter was expressed specifically in the urothelium, and up to 100–500 ng/mL of human growth hormone was found in the resulting urine of mice.**
- **Pig semen contains 30 mg of protein per mL and boars can produce 200–300 mL of semen for a total of 6–9 g of protein per ejaculate.**

Model 4. Silkworm Cocoon

- The **silkworm** has acquired the ability to synthesize **bulk amounts of silk proteins in its silk glands.**
- It can be used for **production of fibrous proteins such as collagens, elastin, and silk.**

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XenoMouse: Production of Fully-Human Monoclonal Antibodies

- In theory, monoclonal antibodies can be **effective agents for diminishing the proliferation of cancer cells and treating other human diseases.**
- However, it is **impossible to generate human monoclonal antibodies.**
- The **rodent monoclonal antibodies are immunogenic to humans** and elicit anti-mouse antibodies that result in destruction of the therapeutic antibody.
- Recombinant DNA strategies have been **devised to "humanize" existing rodent monoclonal antibodies.**
- An antibody is a tetrameric protein with one pair of the heavy chain and one pair of light chain.
- The **genetic information for a specific heavy chain is created by rearrangement of several heavy-chain specific DNA segments in a B cell.** Two **light chains are encoded by DNA rearrangements of other**, light-chain-specific DNA segments.
- **Each single B cell synthesizes only one kind of antibody molecule that has a unique set of rearranged segments for a heavy chain and a light chain.**

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- The genetic repertoire for the **formation of the vast numbers of different human antibodies consists of more than 100 heavy-chain DNA segments and a similar number of light-chain DNA segments.**
- To **create a transgenic mouse that is capable of synthesizing a full range of human antibodies against every antigen, the endogenous mouse heavy and light chain genes were inactivated, and YACs carrying most of the heavy and light-chain DNA elements from each human immunoglobulin gene were inserted into the chromosomal DNA of the mouse.**
- **A commercialized version of the human antibody producing mouse has been designated the XenoMouse.**
- First fully human monoclonal antibody produced in this mouse (**Panitumumab**) has **received regulatory approval for use as a treatment for advanced colorectal cancer.**
- Other therapeutic antibodies produced in the XenoMouse, including several for the treatment of various cancers and osteoporosis, are now in clinical trials.

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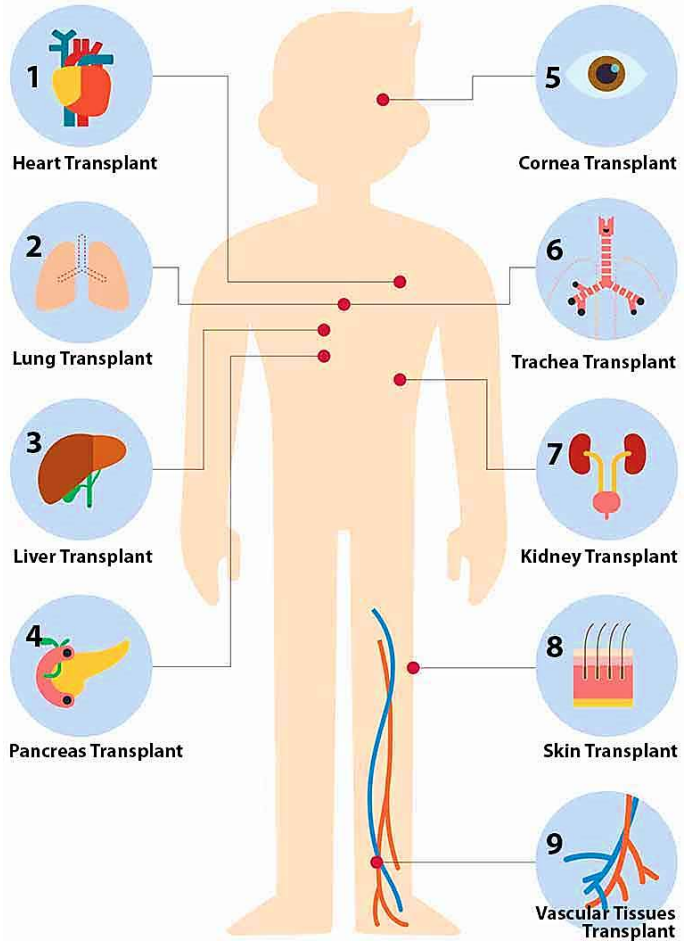
Production of donor organ



Xenotransplantation

- **Xenotransplantation** is defined here as **any procedure that involves the transplantation, implantation, or infusion into a human recipient of live cells, tissues, or organs from a nonhuman animal source.**
- It also **includes any procedure in which human body fluids, cell tissues, or organs have ex vivo contact with live human animal cells, tissues, or organs.**
- More generally, xenotransplantation defines **any cross-species transplantation (e.g., mouse to rat, pig to primate, and sheep to human).**
- **Xenograft - is an organ transplanted from one species to another.**
- In biomedical research, a recent approach to xenotransplantation **targets pigs as source animals** with the goal of **transplanting pig solid organs, such as kidneys, hearts, and livers, into humans.**
- In some cases, **external (ex vivo) pig liver has been used for temporary perfusion for bridging acute liver failure.**
- Among other goals are the use of **encapsulated porcine islet cells for diabetes therapy and the use of fetal porcine neural stem cells for Parkinson cell therapy.**

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Requirements for a successful source animal

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The **pig is generally considered to be the most suitable xenograft** source animal because of its **size, favorable breeding characteristics, pathogen free pig breed are available, pig organs are more or lass similar to that of size of humans and the similarity of many of its organ systems to those of humans and risk of infection is lower in non human primates.**

- **Availability:** Swine are not an endangered species.
- **Safety:** As in the case of allogeneic transplantation, the two most important safety concerns for xenotransplantation are the **risk of failure due to rejection and the risk of side effects due to the immunosuppressive medications** required to prevent rejection, most importantly, infections, to which these medications lower resistance.
- **Size:** One of the attractive features of swine as potential xenograft sources is the fact that their **organs could potentially be matched to the size of any potential human recipient.**
- **Breeding characteristics:** They have large litter sizes (5 to 10 offspring), early sexual maturity (5 months), short gestation time (114 days), and frequent estrus cycles (every 3 weeks).
- **Structural and physiologic similarity to humans:** Most tissues and organs of swine bear a **remarkable resemblance to those of humans both in structure and physiology.** This includes the heart and circulatory system, the kidney, the pancreas, the liver, the lungs, and even the skin, which is almost indistinguishable histologically from the skin of humans.

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Genetically Modified Pigs as Organ Donors for Xenotransplantation

- Genetically modified pigs hold great promise in xenotransplantation. Therefore, **genetically modified pigs can become cell, tissue and organ donors, providing a solution to severe shortage of organ donors.**
- However, transplants from pigs to primates are **subject to vigorous immunologic rejection involving both innate and adaptive immune responses** which results in **graft rejection.** Depending on the mechanism and timing of xenotransplant rejection, the following classification of graft rejection was adopted:
    i. **hyperacute rejection (HAR),**
    ii. **delayed xenograft rejection (DXR),**
    iii. **acute cellular rejection (ACR)**
    iv. **and chronic rejection (CR)**
- Genetic disparities that occurred during evolution resulted in incompatibilities that need to be overcome for xenotransplantation to be successful.
- Fortunately, largely **because of their favorable breeding characteristics, pigs are particularly responsive to engineering of their genome**.
- Enormous progress has been made over the past two decades toward overcoming relevant interspecies incompatibilities.

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Immune barriers to xenotransplantation

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- Transplants from **pigs to primates are subject to vigorous immunologic rejection** involving both innate and adaptive immune responses.
- The innate barriers include monocytes and macrophages, natural killer (NK) cells and complement and coagulation pathways.
- In addition, **Nabs**, which are often classified as innate immune components because they arise without exposure to their known ligands, present **another formidable barrier.**
- The most important of these **Nabs recognizes α-galactose-1,3-galactose (Gal).**
- Gal is a carbohydrate moiety displayed on numerous cell surface glycoproteins and glycolipids of pigs and most other mammalian species, excepting humans and Old World monkeys, due to a frameshift mutation in α-1,3-galactosyltransferase (GalT) in our ancestors.
- The high levels of NAbs to Gal in human serum likely reflect exposure to it on numerous microorganisms.
- Anti-Gal NAbs constitute about 1 to 4% of circulating human immunoglobulin and include both IgM and IgG.
- **Anti-Gal NAbs were a major early obstacle to xenotransplantation.** Antibodies to Gal are responsible for HAR, which occurs within hours after transplant.
- **T cells can directly attack the graft** and can also promote B and NK cell responses. T cell responses—including cytotoxicity, cytokine production, and recruitment and activation of innate cytotoxic cells—are not fully overcome by immunosuppressive therapy in pig-to-primate xenotransplantation.

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Genetic modifications to that have been made in pigs to facilitate pig-to-human organ transplantation

- To obtain transplantable organs from the animal donor, **the Gala(1,3)Gal antigen must be removed from xenograft cell surfaces.** The best method to rid off Gala(1,3)Gal epitopes is **the inactivation of the gene encoding GGTA1, which acts as a catalyst for the Gala(1,3)Gal epitope forming reaction.** Genetic recombination can be used to replace the wild-type GGTA1 gene with a mutant variant, preventing the production of the enzyme.
- Another **xenoreactive antigen found on porcine cell surfaces**, to which humans and non-human primates have antibodies, is a **glycan produced by** the **b1,4-N-acetylgalactosaminyltransferase (b4GalNT2)** enzyme activity. Estrada **used the CRISPR/Cas9 technique to produce pigs lacking the GGTA1, CMAH and b4GalNT2 genes.**
- In vitro tests showed reduced human IgM and IgG binding to PBMCs from genetically modified animals (GGTA1/CMAH/b4GalNT2-inactivated).

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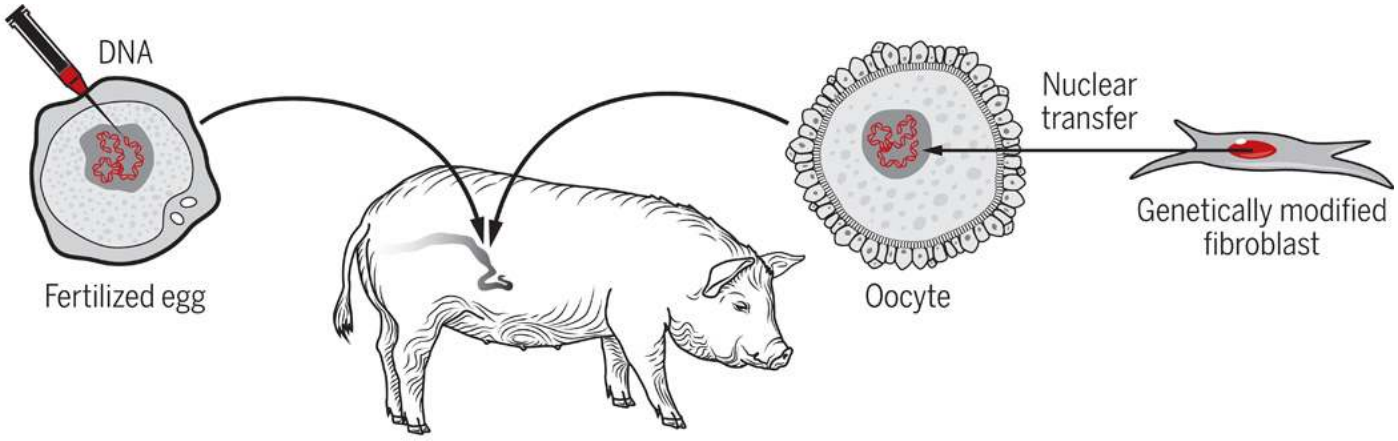
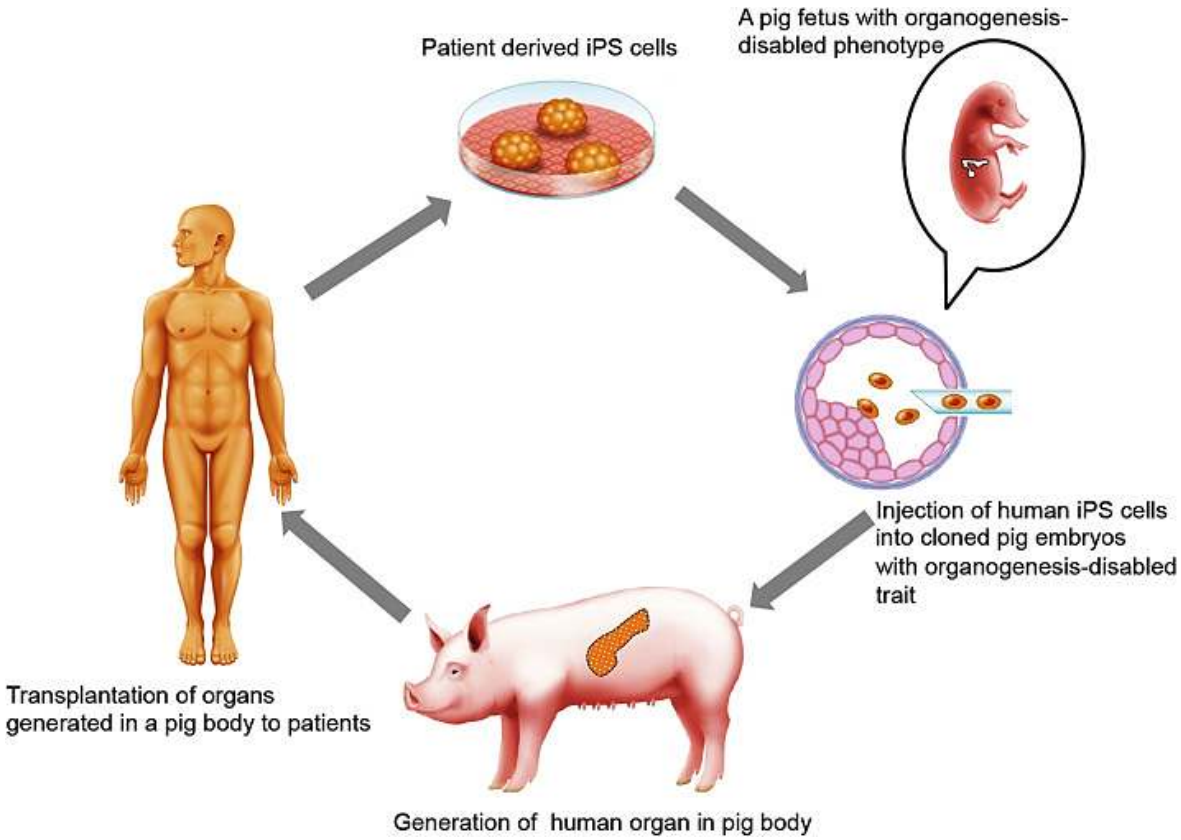
Other Genetic modifications



Transgenics		Knockouts
Complement inhibition	Immunosuppressive molecules	α1,3-galactosyltransferase
hDAF	Anti-CD2	CMAH
hCD46	CTLA4Ig	B4GalNT2
hCD59	hCD47	vWF
		PERVs
Coagulation inhibition	PERV siRNA	
hCD39	MHC genes	
Human thrombomodulin	Class I (NK inhibition)	
Anti-inflammatory genes		
HO-1		
A20		

Generating human tissues and organs from pluripotent stem cells

- **Human organs** with complex physiological functions and three-dimensional structures are **extremely difficult to build in an artificial culturing environment.**
- Therefore, the **use of animal fetuses is a promising alternative strategy that may circumvent the problems** of in vitro organ generation by supporting organ development from xenogeneic PSCs in a natural physiological environment.
- Recently, a new approach using **blastocyst complementation** has been developed and attracted considerable attention.
- It is based on **generating animal fetuses deficient in a specific organ and using the empty space in the fetal body as a niche for the growth and differentiation of allogeneic or xenogeneic PSCs, ultimately to form a solid organ.**



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Conceptus complementation using pig fetuses with organogenesis-disabled phenotype

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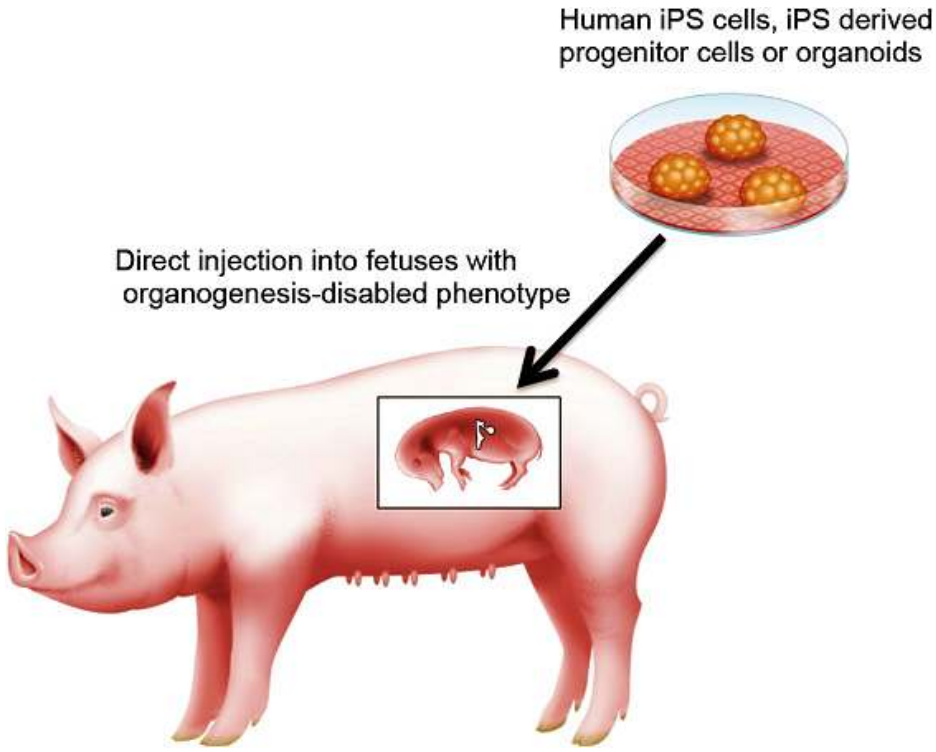

Porcine fetuses with "organ niche," that is, organogenesis-disabled phenotype are expected to be useful to grow human organ progenitors or anlagen.

- Pigs with the **apancreatic phenotype due to *Pdx1-Hes1* overexpression** or *Pdx1* deficiency show neonatal lethality.
- The **"organ niche" here was created by knocking out the host's master regulator of Pancreas development (the Pdx1 gene)** with **donor PSC-derived cells and then "complementing" this deficiency to generate a newly formed pancreas composed entirely of injected cells.**
- These complemented **embryos survived to adulthood without abnormalities**, indicating that the PSC-derived pancreas was functional.
- When the **loss of a specific organ is not fatal for the fetus**, as is the case with the **pancreatogenesis-disabled phenotype** in the fetal development may be effective in **generating the missing organ from exogenous cells.**

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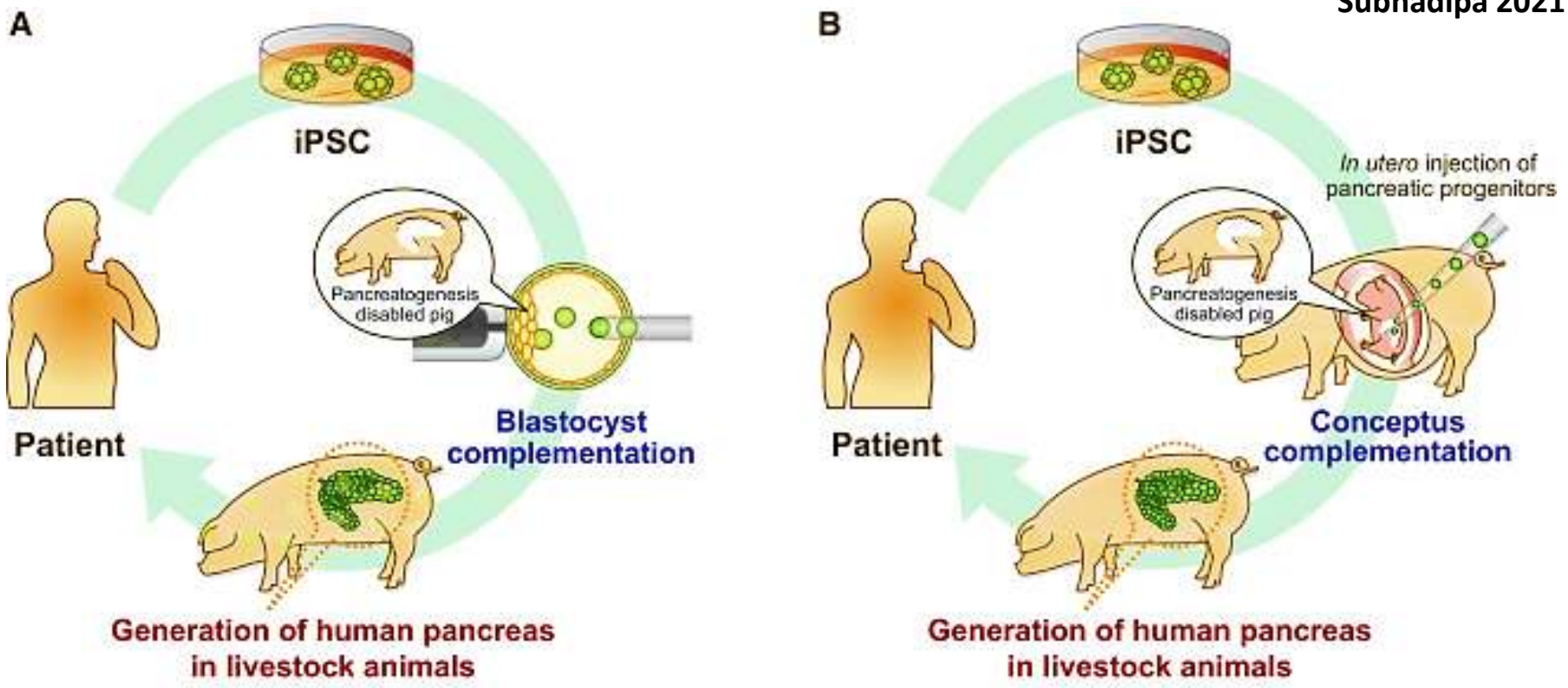

(A) Generation of human organs using blastocyst complementation of organogenesis-disabled livestock.

(B) Generation of human organs using in utero conceptus complementation of organogenesis-disabled livestock.

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