Microbes in Medical Biotechnology

Introduction

- The staggering breakthroughs in medical and microbial biotechnology, notably in molecular biology, have led to great strides in the understanding and treatment of human diseases.
- Monoclonal antibodies, gene probes and polymerase chain reaction offer improved diagnosis of infectious diseases and other ailments.
- Microbial and animal host cells, transgenic animals and plants generate genetically-engineered, high value pharmaceuticals and vaccines in large quantity.
- Sophisticated structural studies and computer modeling of complex molecules permit the design of novel drugs. Previously intractable infections are now preventable by novel immunization strategies using recombinant DNA technology, and amenable to new anti-microbial agents discovered from nature.
- Complemented by the enormous Human Genome Project, gene therapy is at the threshold of a new dimension in medical science.
- Recognizing the impact of these advances on human health and economic development, scientists are harnessing these enabling technologies to meet the new challenges in medicine, including the disciplines of medical microbiology and infectious diseases.

Applications

- Pharmacology:
  - Insulin production
  - Human growth hormone
  - Human blood clotting factor
  - Gene pill
  - Monoclonal antibodies
- Gene therapy
- Stem cells
- Tissue engineering

Recombinant proteins

1.**Human Growth Hormone** : somatotropin or somatropin, is a peptide hormone that stimulates growth, cell reproduction and regeneration.

2. **Insulin** : Insulin is a hormone made by the pancreas that allows your body to use sugar (glucose) from carbohydrates in the food.

3. **Follicle Stimulating Hormone** : a hormone secreted by the anterior pituitary gland which promotes the formation of ova or sperm.

4. **Erythropoietin** : a hormone secreted by the kidneys that increases the rate of production of red blood cells in response to falling levels of oxygen in the tissues.

5. **Tissue Plasminogen Activator** : Tissue plasminogen activator is a protein involved in the breakdown of blood clots.

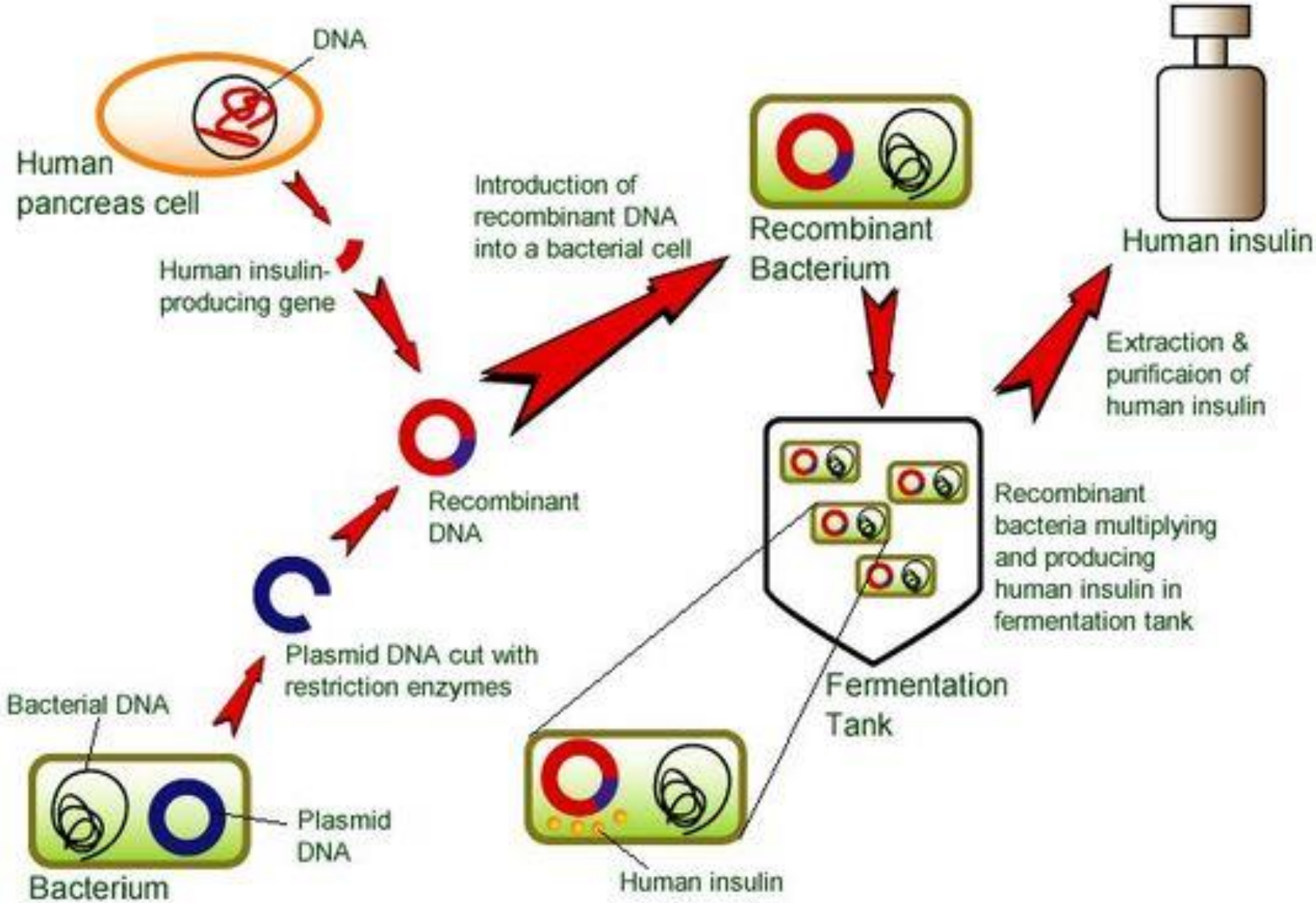
6. **Factor VIII** : Essential blood clotting protein

Pharmacology

Insulin production:

- The production of genetically engineered human insulin was one of the first breakthroughs of biotechnology in the pharmaceutical industry.
- Insulin was first produced in *Escherichia coli* through recombinant DNA technology in 1978.
- Process: the human gene for the insulin is placed into bacteria, are cultured and allowed to produce insulin which is collected, purified and sold to diabetic people worldwide.
- Besides, *Saccharomyces cerevisiae*, an eukaryotic microbial system is also used for the expression of the protein.
- Advantages of *E. coli*: simple genetics, cost effective, fast expression (doubling time of E.coli ~ 20-30 mins), well established protocols, ease of scaling up the fermentation process, ease of purification.
- Advantages of *S. cerevisiae*: non-pathogenic, rapid growth (generation time ~ 80min), dispersed cells, ease of replica plating and mutant isolation, grown on defined media, well defined genetic system, high versatile DNA transformation system.

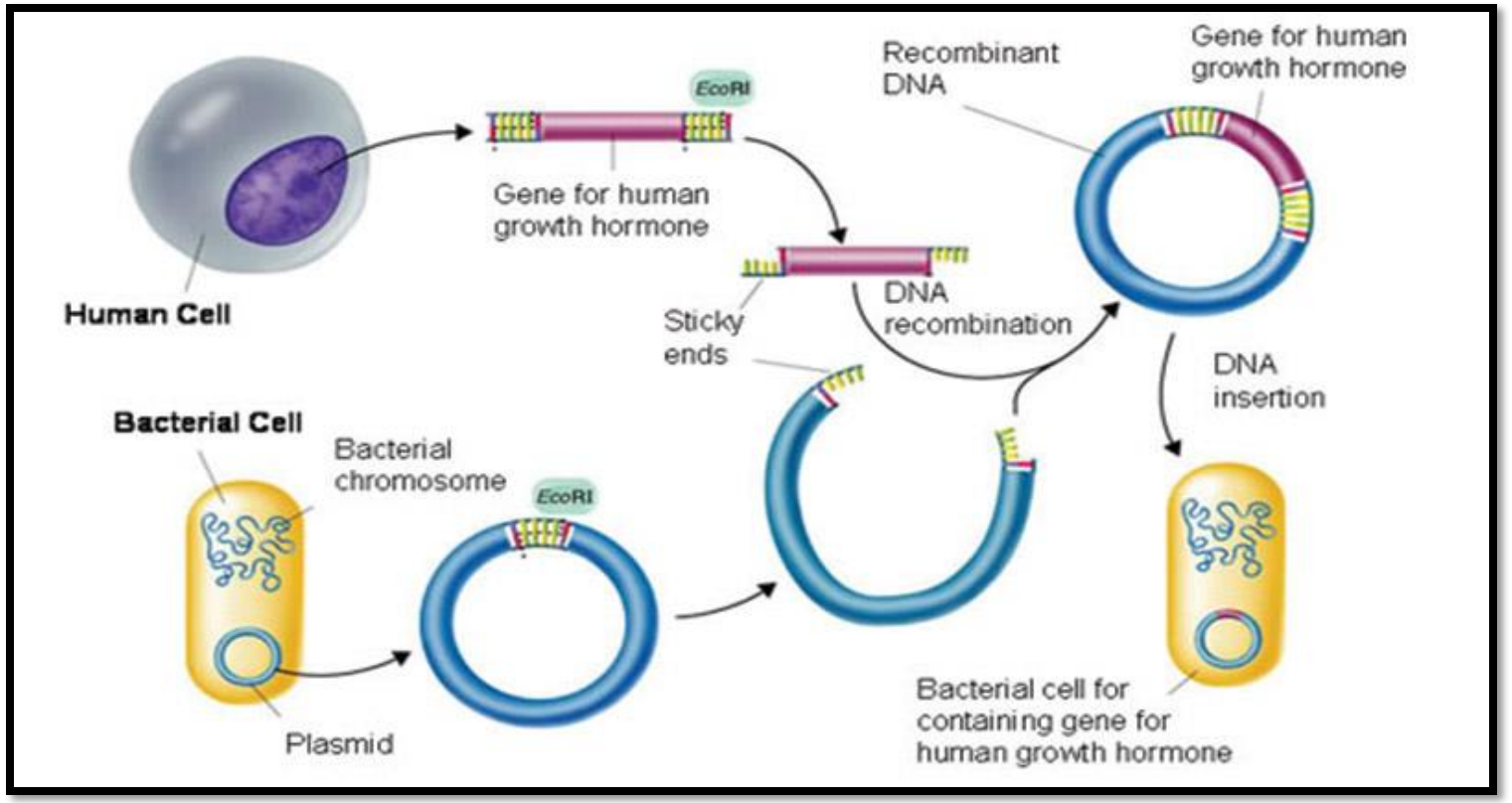
Human Insulin Production



Pharmacology

Human growth hormone:

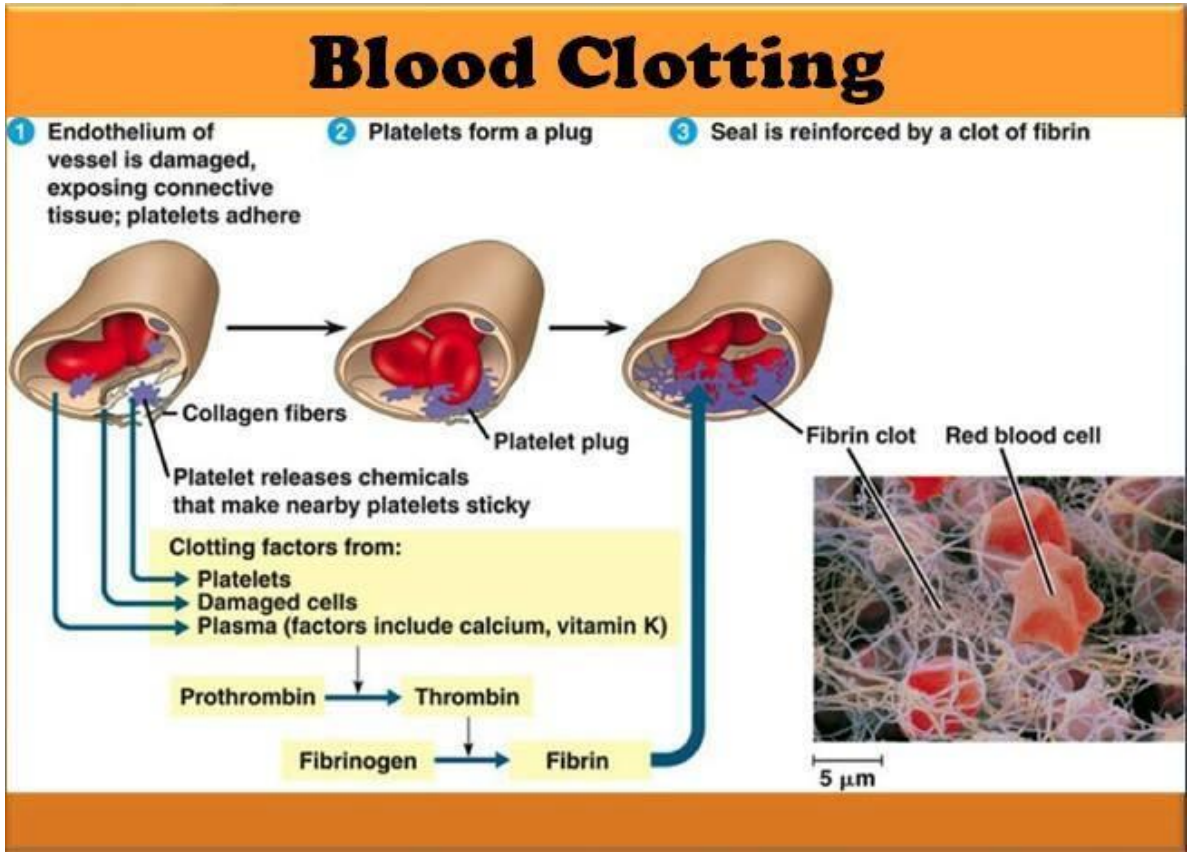
- Production of the human growth hormone was first done in 1979 using recombinant DNA technology.
- Scientist produced human growth hormone by inserting DNA coding for human growth hormone into a plasmid that was implanted in *E. coli*.
- This gene was created using reverse transcription of the mRNA found in the pituitary glands to complementary DNA.
- Prior to this development, human growth hormone was extracted from the pituitary glands of the cadavers, as animal growth hormones have no therapeutic values in humans.

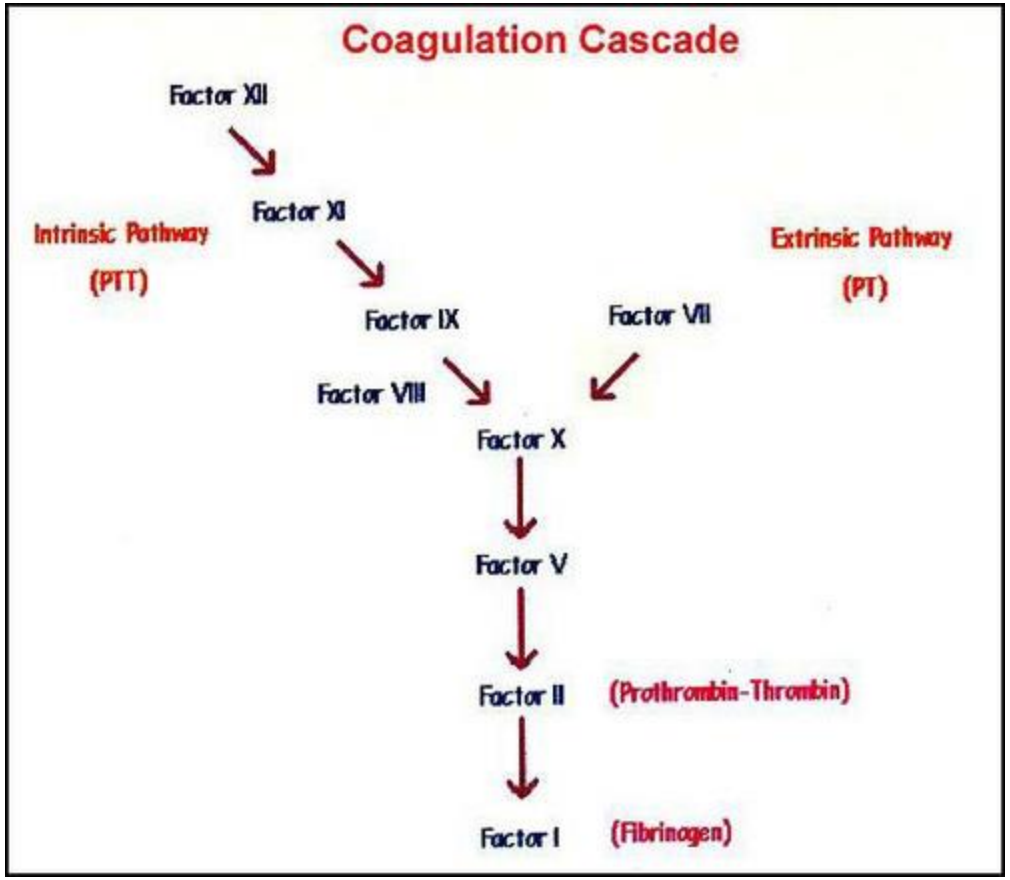


Pharmacology

Human blood clotting factor:

- Production of human clotting factor was enhanced through recombinant DNA technology.
- It is the first recombinant product obtained using Chinese hamster ovary cells in 1986.
- Plasmids containing the factor IX gene, along with the plasmids with a gene that codes for resistance to methotrexate, were inserted into CHO cells via transformation.





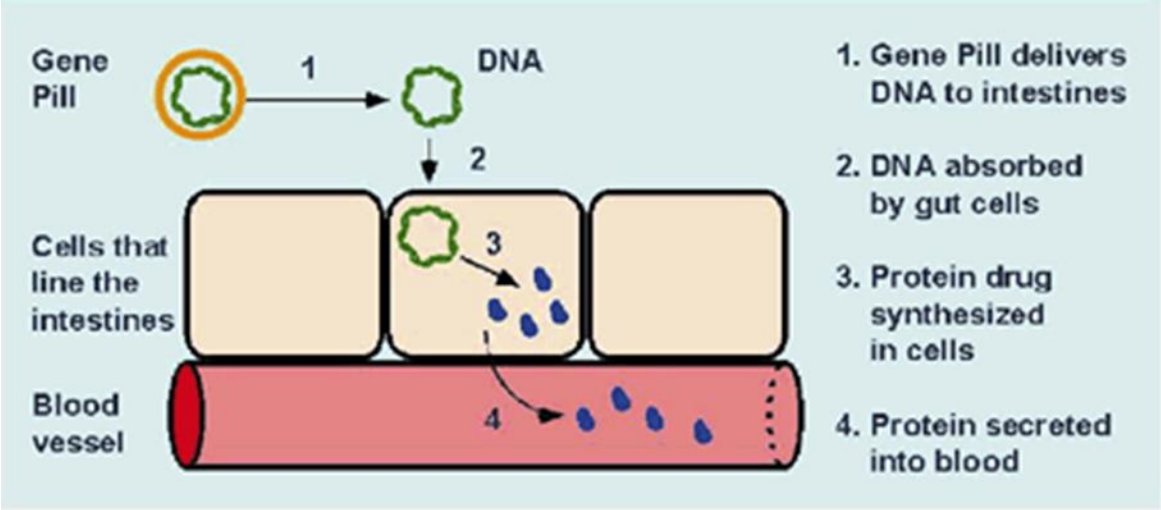
Pharmacology

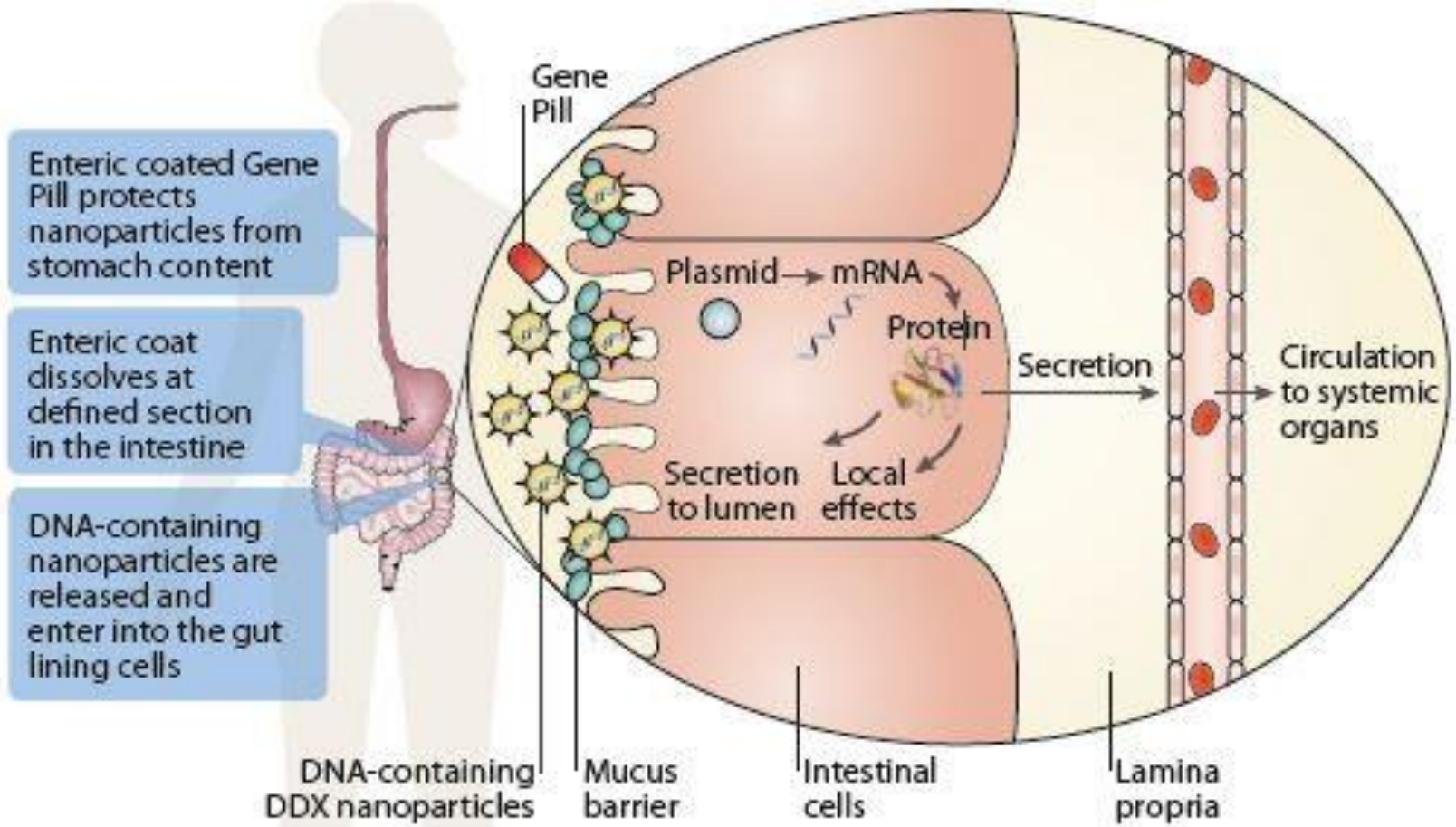
Gene pill:

- Gene delivery represents a revolutionary therapeutic approach with the potential for sustained protein production by the human body, leading to a convenient and effective method for systemic delivery of protein drugs.
- The Gene Pill enables DNA delivery in a non-invasive manner, leading to the secretion of therapeutic proteins into a patient's blood, supplanting the need for injection of therapeutic protein products.
- The Gene Pill also has potential for the development of oral DNA vaccination through expression of protein antigens in the gut lymphoid tissue.
- This approach limits the biodistribution of the delivered DNA to the gut and retains all of the safety advantages of non-viral gene delivery, including repeat dosing.

Gene pill

- A breakthrough in the delivery of gene therapy was announced by Genteric, Inc. (http://www.genteric.com).
- Called the 'Gene Pill', it is a technique for oral delivery of non-viral DNA. The idea behind the pill is that the gastrointestinal organs will convert the introduced DNA to therapeutic proteins that will be distributed naturally by the body.
- The company tested the method using DNA encoding the human insulin gene.
- Studies showed that when the engineered DNA is introduced into the body, it is resistant to degradation. When tested in diabetic rats, insulin protein was produced and secreted into the blood stream, lowering the rats' blood glucose levels to normal.
- The technology has the potential to treat a wide array of other diseases such as growth hormone deficiency.





The Gene Pill. This system turns intestinal cells into 'bioreactors' that produce therapeutic proteins for local effects or for potential secretion into the circulation for systemic effects.

enGene has developed a proprietary nonviral gene delivery platform and Gene Pill technology to deliver orally administered gene therapies to mucosal tissues, creating new opportunities for treating hard-to-reach tissues across many indications.

Pharmacology

Monoclonal antibodies:

- Monoclonal antibodies (mAbs) are monovalent (one epitope) antibodies derived from clones of a single parental immune cell.
- The original method for producing monoclonal antibodies is found in hybridoma technology.
- Prior to the production of monoclonal antibodies, polyclonal antibodies (multiple epitopes, multiple parental cells) were the standard for *in vitro* research. Production of polyclonal antibodies, however, was too inconsistent to be an effective tool for *in vivo* research.
- The breakthrough came in the 1970s, when Kohler and Milstein succeeded in creating the first hybridoma cell line. They were awarded the Nobel Prize for Medicine and Physiology in 1984.

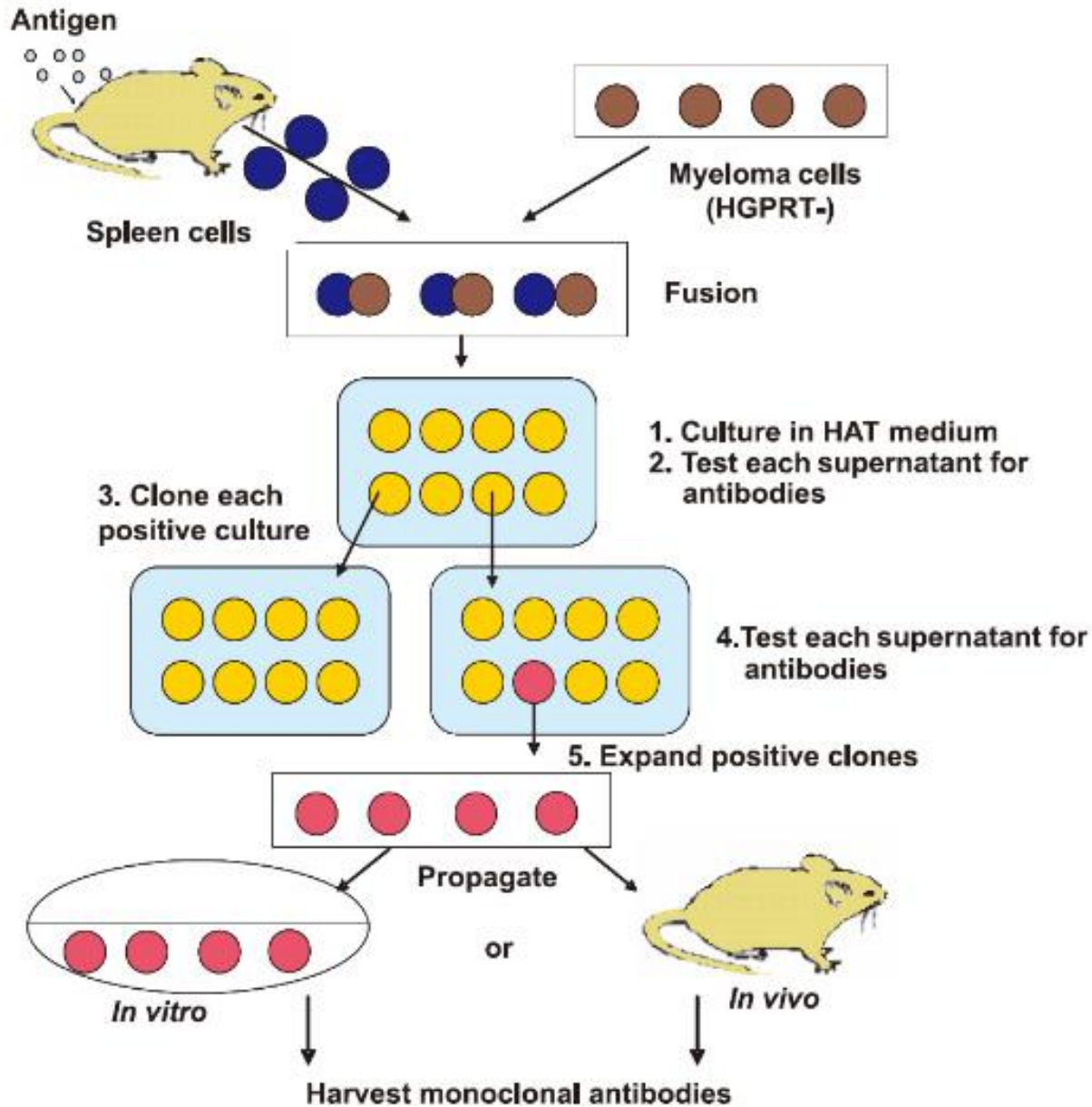
- Hybridoma technology involves immunizing a host animal with an antigen of interest to elicit an immune response. When a sufficient response is achieved as determined by *in vitro* testing, the mature B-cells are harvested from the animal spleens. The B-cells are then fused with myeloma cells using polyethylene glycol (PEG) to generate an immortalized hybridoma cell line. Through multiple rounds of screening, a single, pure, hybridoma positively-expressing the antibody against the antigen of interest, as determined by ELISA, is amplified and monoclonal antibodies are purified.

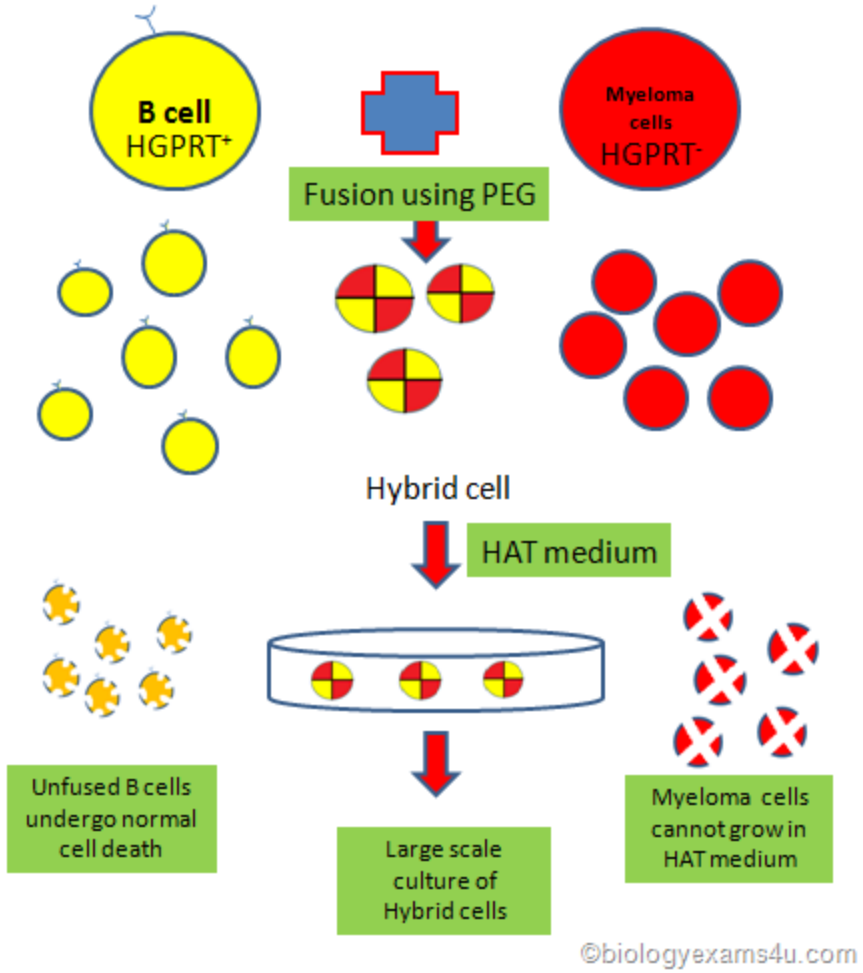
The hybridoma technology of monoclonal antibody production:

1. Cell fusion
    - Polyethylene glycol (PEG) and electrofusion are commonly used to induce cell fusion in hybridoma production.
    - PEG fuses the plasma membranes of adjacent myeloma and/or antibody-secreting cells, forming a single cell with two or more nuclei.
    - This heterokaryon retains these nuclei until the nuclear membranes dissolve before mitosis.
    - Electrofusion joins the membranes of neighboring cells by the application of a pulsed electrical field.
    - Electrofusion is more efficient than PEG and the results are reproducible.
2. Hybridoma screening
    - Commonly, the myeloma cells have a defective HGPRT enzyme (hypoxanthine-guanine phosphoribosyl transferase), blocking their ability to use the salvage pathway.
    - These cells containing a non-functional HGPRT protein will die in HAT medium.
    - Only the hybridoma cells have got the ability to divide and proliferate on the HAT medium because genome from the B-lymphocyte makes them HGPRT positive and genome from the myeloma cells they can divide indefinitely.
3. mAb production
    - Hybridoma antibodies can be produced in vitro and in vivo.

- For production of monoclonal antibodies ***in vitro***, hybridomas are expanded by transfer to 24 well tissue culture plates followed by 25 cm^2 flask and a 75 cm^2 flask containing suitable medium.
- The cell density is maintained between 10^5 and 10^6 cells/ml.
- Typical culture supernatants yield up to l00µg/ml of antibody, the exact amount depending upon the cell density and rate of growth.
- Culture in vitro provides a more pure preparation of antibody.

- For producing monoclonal antibodies ***in vivo***, mice are primed by intraperitoneal injection with 10^5 - 10^7 hybridoma cells.
- The rate of growth of the resulting ascites tumor is in general very variable and can be from less than two or more than five weeks. (Ascites; the accumulation of fluid in the peritoneal cavity, causing abdominal swelling)
- The ascites fluid can be collected from an anaesthetized mouse.
- It is possible to obtain 10 ml of ascites fluid or more from a mouse by regular tapping.
- Ascites fluid will be contaminated with mouse immunoglobulins to a small extent and if a very pure antibody is required this may prove inconvenient.


Antigen
Spleen cells
Myeloma cells
(HGPRT-)
Fusion
1. Culture in HAT medium
2. Test each supernatant for
antibodies
3. Clone each
positive culture
4.Test each supernatant for
antibodies
5. Expand positive clones
Propagate
or
In vitro
In vivo
Harvest monoclonal antibodies



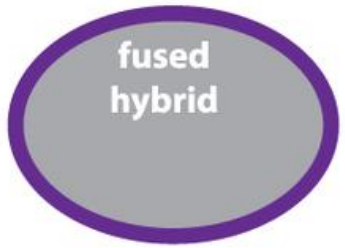
HAT MEDIUM: A selection medium for hybrid cell lines; contains **hypoxanthine; aminopterin; thymidine**. Only cell lines expressing both hypoxanthine phosphoribosyl transferase (HPRT+) and thymidine kinase (TK+) can survive in this medium.

The purpose of the medium is to: (1) selectively kill unfused myeloma cells that are well adapted to tissue culture and would otherwise outgrow any hybridomas produced and (2) eliminate any myeloma-myeloma hybridomas that lack HPRTase.

Hybridomas are thus selected by continuously feeding the mixture of the three different cell types with HAT medium (kept in 96-well microtiter plates in a carbon dioxide incubator at 37°C): Unfused plasma cells are easily eliminated in these conditions since they do not replicate in culture medium.

When two types of cells, one with a mutation in TK and the other with a mutation in HGPRT are fused, only the hybrid cells will contain the full complement of necessary enzymes for growth on HAT medium via the salvage pathway. Thus only hybrid cells will grow in HAT medium.

HAT Selection

Genotype:*	**TK -**	**TK+/TK -**	**TK +**
Cell type:			
HAT fate:	**DIES**	**SURVIVES**	**DIES**
Explanation:	**Unable to synthesize DNA:** (1) Thymidine kinase* mutation causes a loss-of-function in the "salvage" pathway and (2) Aminopterin blocks "De novo" pathway.	**Immortal and restored DNA synthesis:** (1) Immortality from plasmacytoma and (2) rescued ability to synthesize DNA due to restored thymidine kinase* function.	**Mortal:** (1) Functional DNA synthesis, but (2) eventually dies because of limited number of replication cycles

**HGPRT (hypoxanthine-guanine phosphoribosyltransferase) mutants can be used in place of TK (thymidine kinase) mutants*

APPLICATION OF HYBRIDOMA TECHNOLOGY

- **Serological**;
  - Identification of ABO blood group
- **Diagnosis**:
  - Detection of pregnancy by assaying of hormones with monoclonal.
  - Separation of one substance from a mixture of very similar molecules.
- **Immunopurification**:
  - Purification of individual interferon using monoclonal
  - Inactivation of T-lymphocytes responsible for rejection of organ transplants.
- **Therapy**:
  - Removal of tumor cell from bone marrow.
  - Treatment of acute renal failure.
  - Treatment malignant leukemic cells, B cell lymphomas, and a variety of allograft rejections after transplantation.

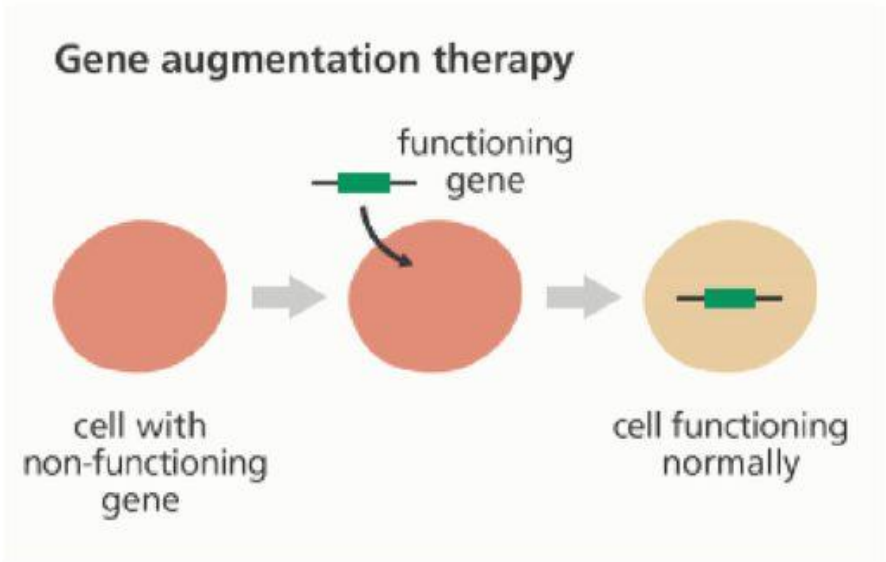
Gene therapy

- Gene therapy is an experimental technique that uses genes to treat or prevent disease. In the future, this technique may allow doctors to treat a disorder by inserting a gene into a patient's cells instead of using drugs or surgery. Researchers are testing several approaches to gene therapy, including:
  - Replacing a mutated gene that causes disease with a healthy copy of the gene.
  - Inactivating, or "knocking out," a mutated gene that is functioning improperly.
  - Introducing a new gene into the body to help fight a disease.
- Although gene therapy is a promising treatment option for a number of diseases (including inherited disorders, some types of cancer, and certain viral infections), the technique remains risky and is still under study to make sure that it will be safe and effective. Gene therapy is currently being tested only for diseases that have no other cures.
- There are two different types of gene therapy depending on which types of cells are treated:
  - **Somatic gene therapy:** transfer of a section of DNA to any cell of the body that doesn't produce sperm or eggs. Effects of gene therapy will not be passed onto the patient's children.
  - **Germline gene therapy:** transfer of a section of DNA to cells that produce eggs or sperm. Effects of gene therapy will be passed onto the patient's children and subsequent generations.

Gene therapy techniques

- There are several techniques for carrying out gene therapy. These include:
- **Gene augmentation therapy**
  - This is used to treat diseases caused by a mutation that stops a gene from producing a functioning product, such as a protein.
  - This therapy adds DNA containing a functional version of the lost gene back into the cell.
  - The new gene produces a functioning product at sufficient levels to replace the protein that was originally missing.
  - This is only successful if the effects of the disease are reversible or have not resulted in lasting damage to the body.
  - For example, this can be used to treat loss of function disorders such as cystic fibrosis by introducing a functional copy of the gene to correct the disease.

1. Gene augmentation therapy



- *This is used to treat disease caused by a mutation that stops a gene from producing a functioning product*

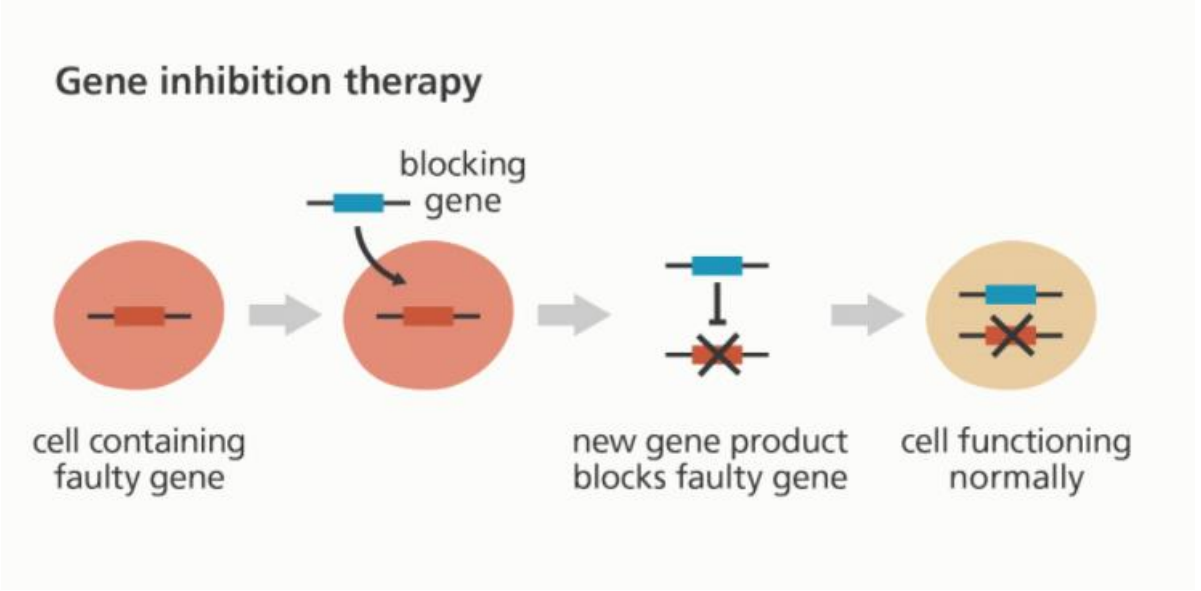
- *This therapy adds DNA containing a functional version of the lost gene back into the cell*

http://www.yourgenome.org/facts/what-is-gene-therapy

Gene inhibition therapy

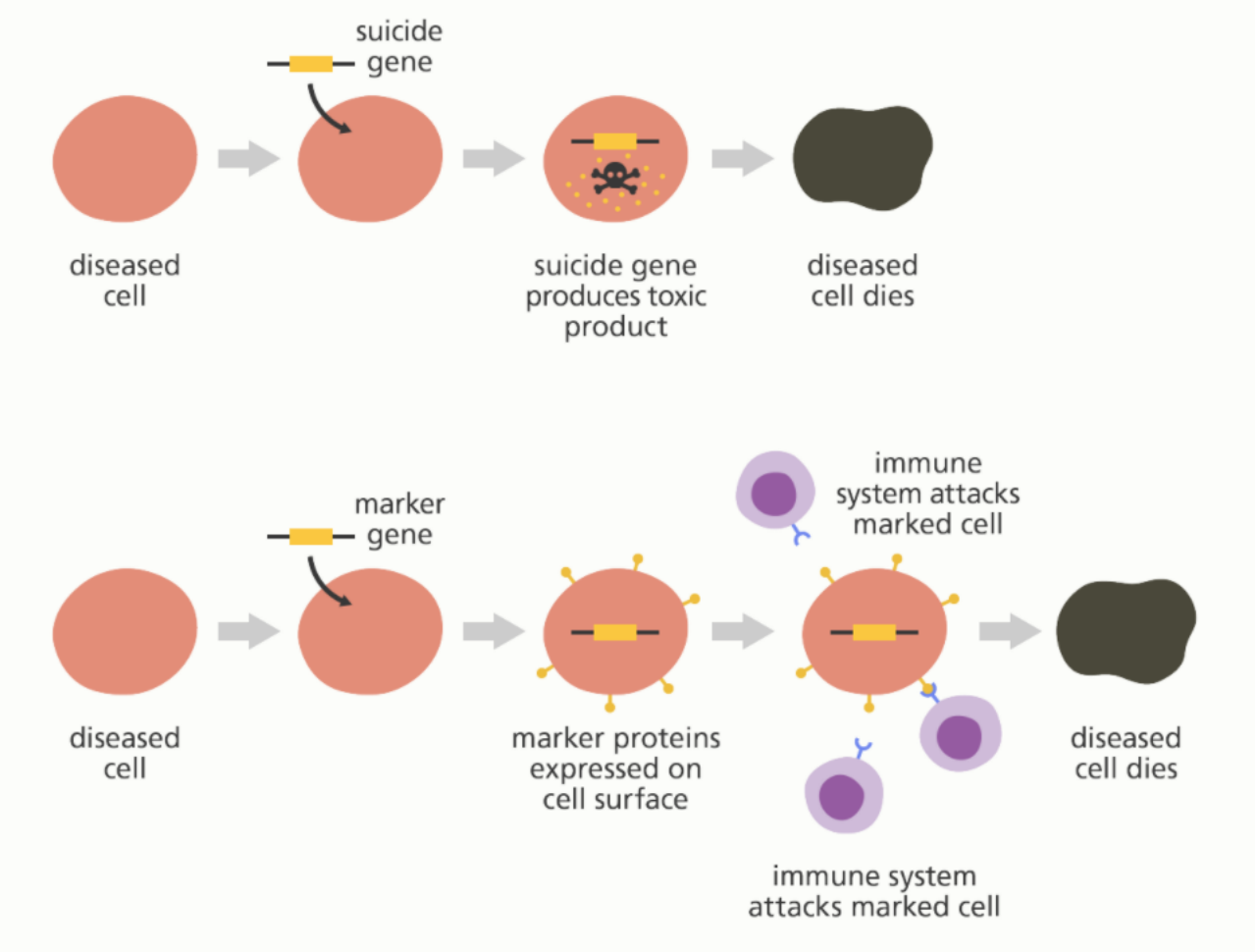
- Suitable for the treatment of infectious diseases, cancer and inherited disease caused by inappropriate gene activity.
- The aim is to introduce a gene whose product either:
- inhibits the expression of another gene
- interferes with the activity of the product of another gene.
- The basis of this therapy is to eliminate the activity of a gene that encourages the growth of disease-related cells.
- For example, cancer is sometimes the result of the over-activation of an oncogene? (gene which stimulates cell growth).

  So, by eliminating the activity of that oncogene through gene inhibition therapy, it is possible to prevent further cell growth and stop the cancer in its tracks.



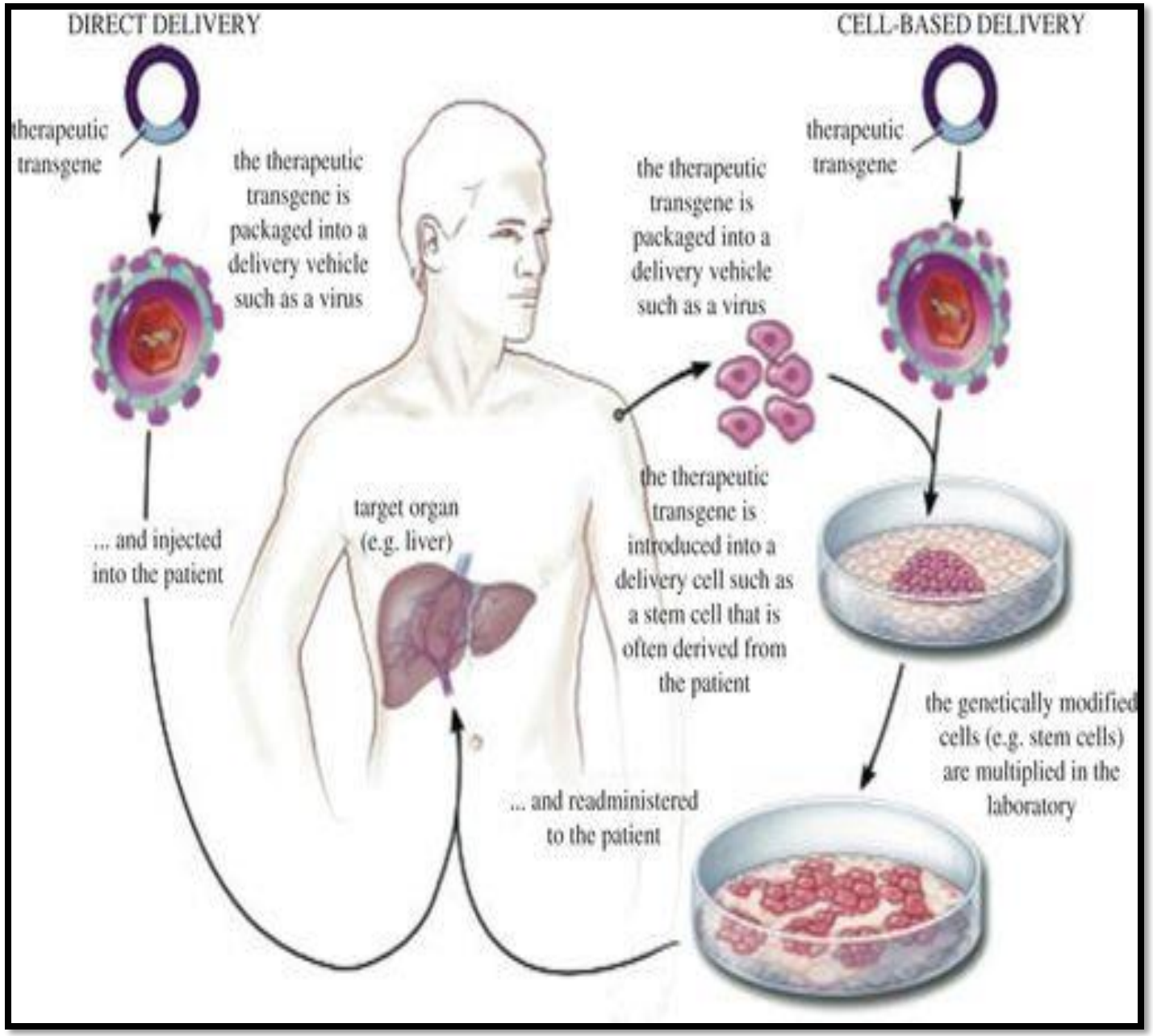
Killing of specific cells

- Suitable for diseases such as cancer that can be treated by destroying certain groups of cells.
- The aim is to insert DNA into a diseased cell that causes that cell to die.
- This can be achieved in one of two ways:
  - the inserted DNA contains a "suicide" gene that produces a highly toxic product which kills the diseased cell
  - the inserted DNA causes expression of a protein that marks the cells so that the diseased cells are attacked by the body's natural immune system.
- It is essential with this method that the inserted DNA is targeted appropriately to avoid the death of cells that are functioning normally.



How is DNA transfer done?

- A section of DNA/gene containing instructions for making a useful protein is packaged within a vector, usually a virus, bacterium or plasmid.
- The vector acts as a vehicle to carry the new DNA into the cells of a patient with a genetic disease.
- Once inside the cells of the patient, the DNA/gene is expressed by the cell's normal machinery leading to production of the therapeutic protein and treatment of the patient's disease.
- Classified as:
  - Direct delivery
  - Cell based delivery



Gene therapy strategies have been developed to treat a wide variety of acquired diseases like cancer, Parkinson's disease, Huntington's disease, influenza, HIV, Hepatitis. Some examples are:

- **Severe combined immune deficiency (SCID):** also known as the ADA-SCID/the bubble boy disease. Affected children are born without an effective immune system and succumbs to infections outside the bubble without bone marrow transplantation from matched donors. The therapeutic gene called ADA was introduced into the bone marrow cells of such patients in the laboratory, followed by transplantation of the genetically corrected cells back to the same patients. The immune system was reconstitutes in all six treated patients without noticeable side effects and now lead a normal life without the need for further treatment.
- **Haemophilia:** patients are not able to induce blood clots and suffer from external and internal bleeding that can be life threatening. The therapeutic gene was introduced into the liver od patients who then acquired the ability to have normal blood clotting time.
- **Chronic granulomatous disorder (CGD):** a genetic disease in the immune system that leads to the patients inability to fight off bacterial and fungal infections that can be fatal. Using similar techniques as in the ADA-SCID trial, investigators in Germany treated two patients suffering from this disease, whose reconstituted immune systems have since been able to provide full protection against microbial infections for at least two years.