RECOMBINANT DNA TECHNOLOGY

II M.SC., MICROBIOLOGY

SEMESTER III

UNIT V (APPLICATIONS OF RECOMBINANT DNA TECHNOLOGY)

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TRICHY - 24

RECOMBINANT DNA TECHNOLOGY

GENE THERAPY

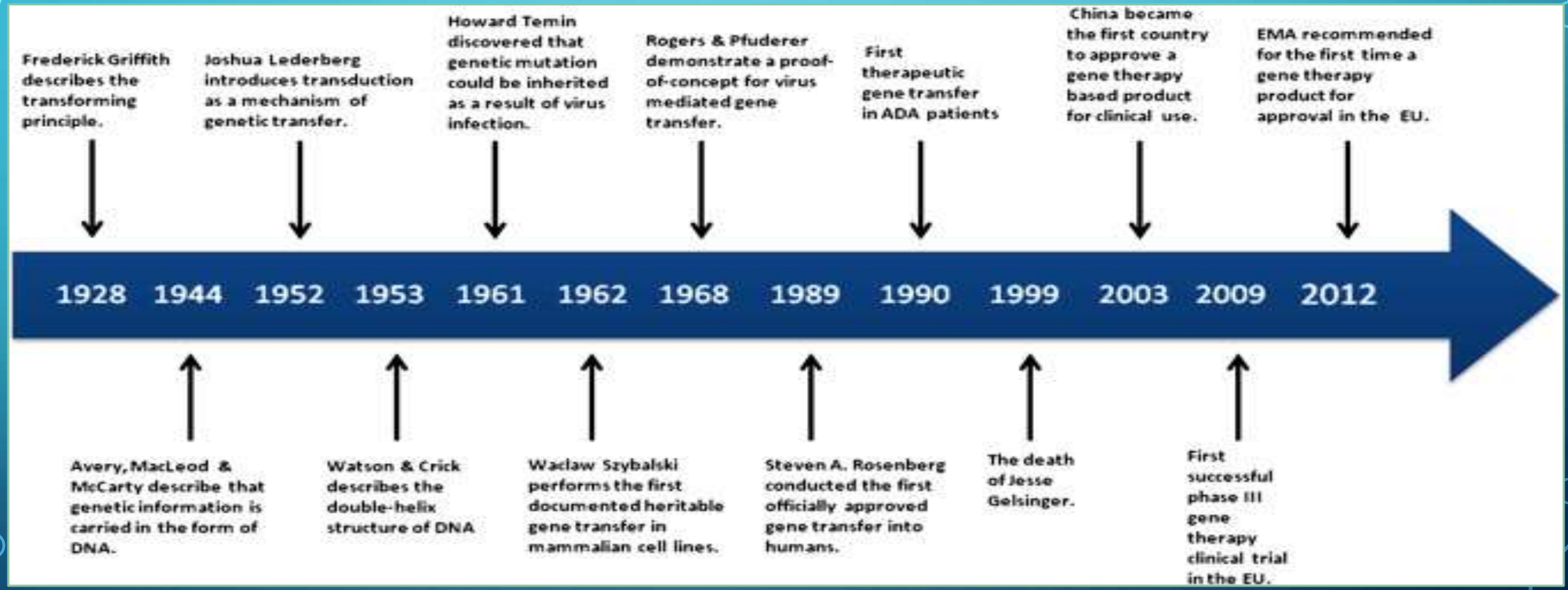
INTRODUCTION

- Gene therapy is a therapeutic technique that uses genes to treat or prevent diseases.
- Gene therapy involves altering the genes inside our body's cells in an effort to trat or stop diseases although not all medical procedures that introduce alterations to a patients genetic makeup can be considered gene therapy .

Various approaches to gene therapy include

- Replacing a mutated gene that causes diseases with a healthy copy of the gene.
- Inactivating or knocking out or fixing a mutated gene that is functioning improperly
- Introducing a new gene into the body to help fight adiseases

HISTORY OF GENE THERAPY



TYPES OF GENE THERAPY

- There are several approaches for correcting faulty genes; the most common being the insertion of a normal gene into a specific location within the genome to replace a non functional gene. Gene therapy is classified into the following two types:

1. Somatic gene therapy
2. Germ line gene therapy

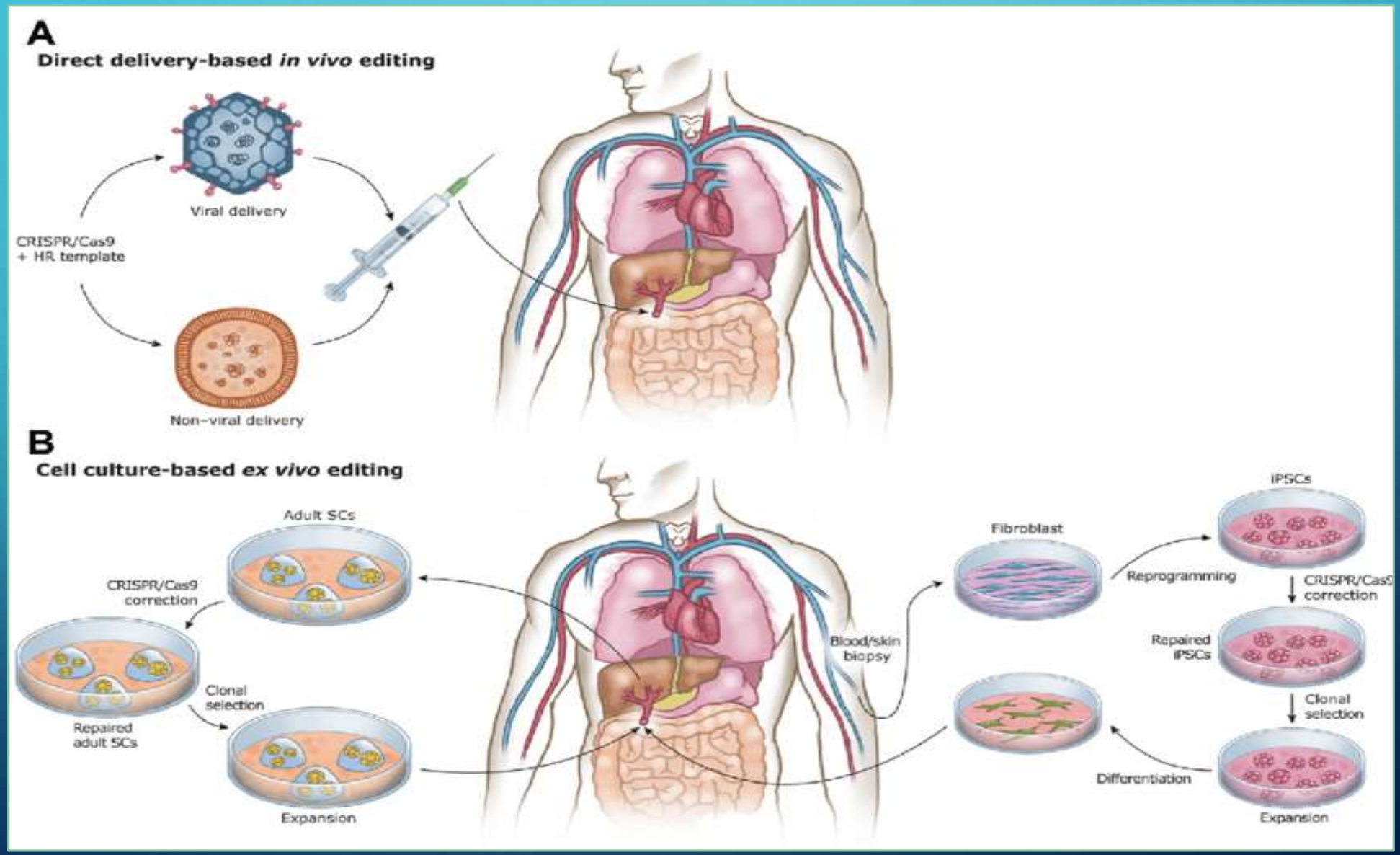
SOMATIC GENE THERAPY

- In somatic gene therapy, the somatic cells of a patient are targeted for foreign gene transfer. In this case the effects caused by the foreign gene is restricted to the individual patient only, and not inherited by the patient's offspring or later generations.

GERM LINE GENE

- Therapy Here, the functional genes, which are to be integrated into the genomes, are inserted in the germ cells, i.e., sperm or eggs. Targeting of germ cells makes the therapy heritable

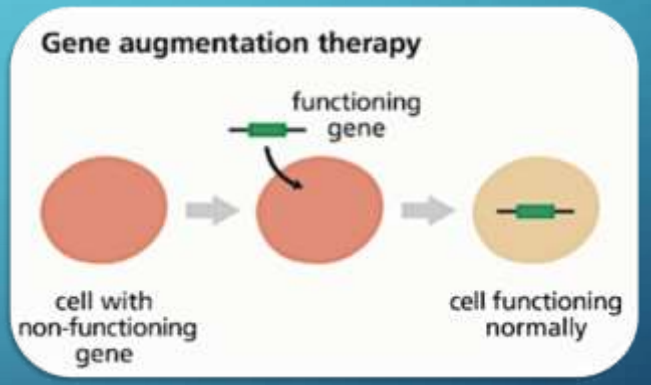
IN VIVO AND *EX VIVO* GENE THERAPY



STRATEGIES OF GENE THERAPY

GENE AUGMENTATION THERAPY

- It is used to treat diseases caused by amutation tht 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 produce a functioning product at sufficient levels to replace the protein that was originally missing.
- This is only successful if the effects of the diseases 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 diseases.



TARGETED KILLING OF SPECIFIC CELLS

- Artificial cell killing and immune system assisted cell killing have been popular in the treatment of cancers.
- The aim is to insert DNA into a diseased cell that causes that cell to die.
- Genes are directed to the target cells and then expressed so as to cause cell killing.

DIRECT KILLING

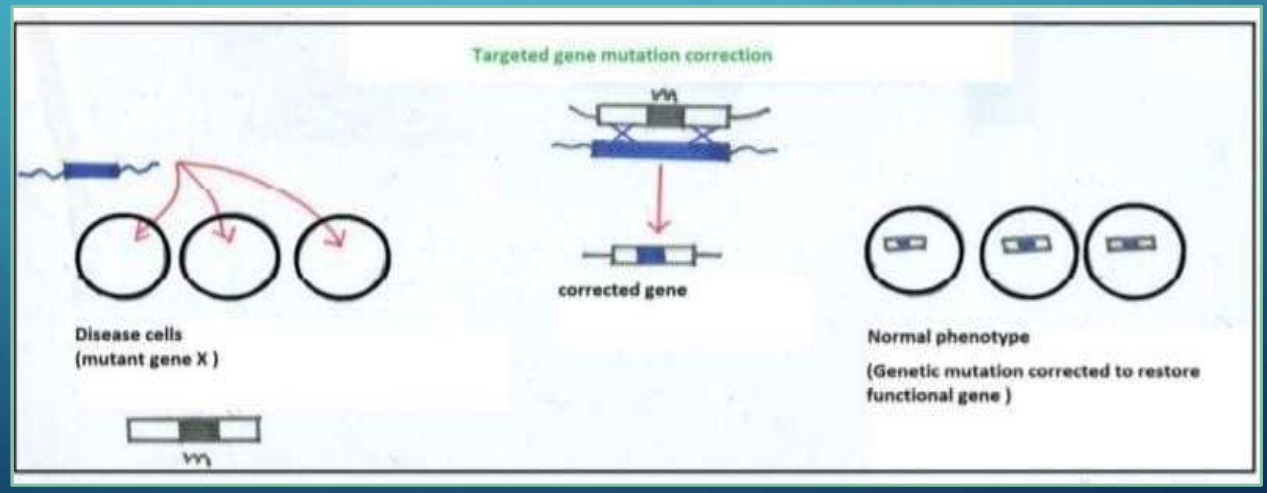
The inserted DNA contains a suicide gene that produce a highly toxic product which kills the diseased cell.

INDIRECT KILLING

Uses immune stimulatory gene to provoke or enhance an immune response against the target cell.the inserted DNA causes expression of a protein that marks the cells so that the diseased cells are attracked 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.

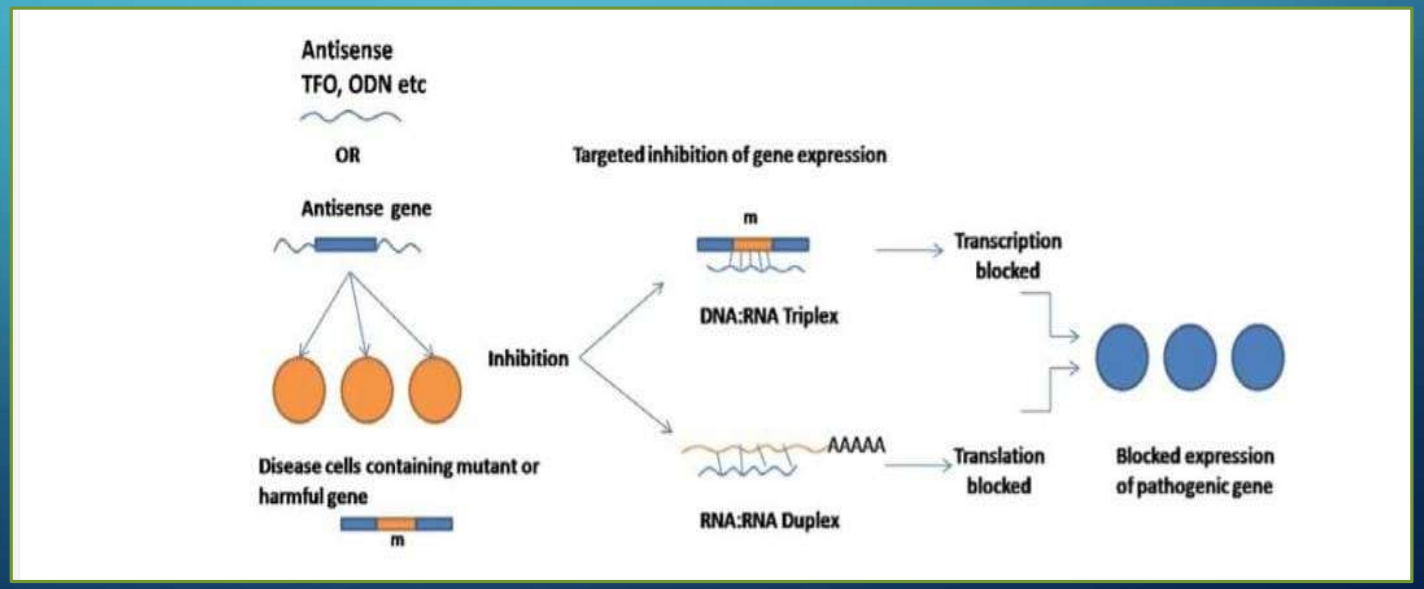
TARGETED MUTATION CORRECTION

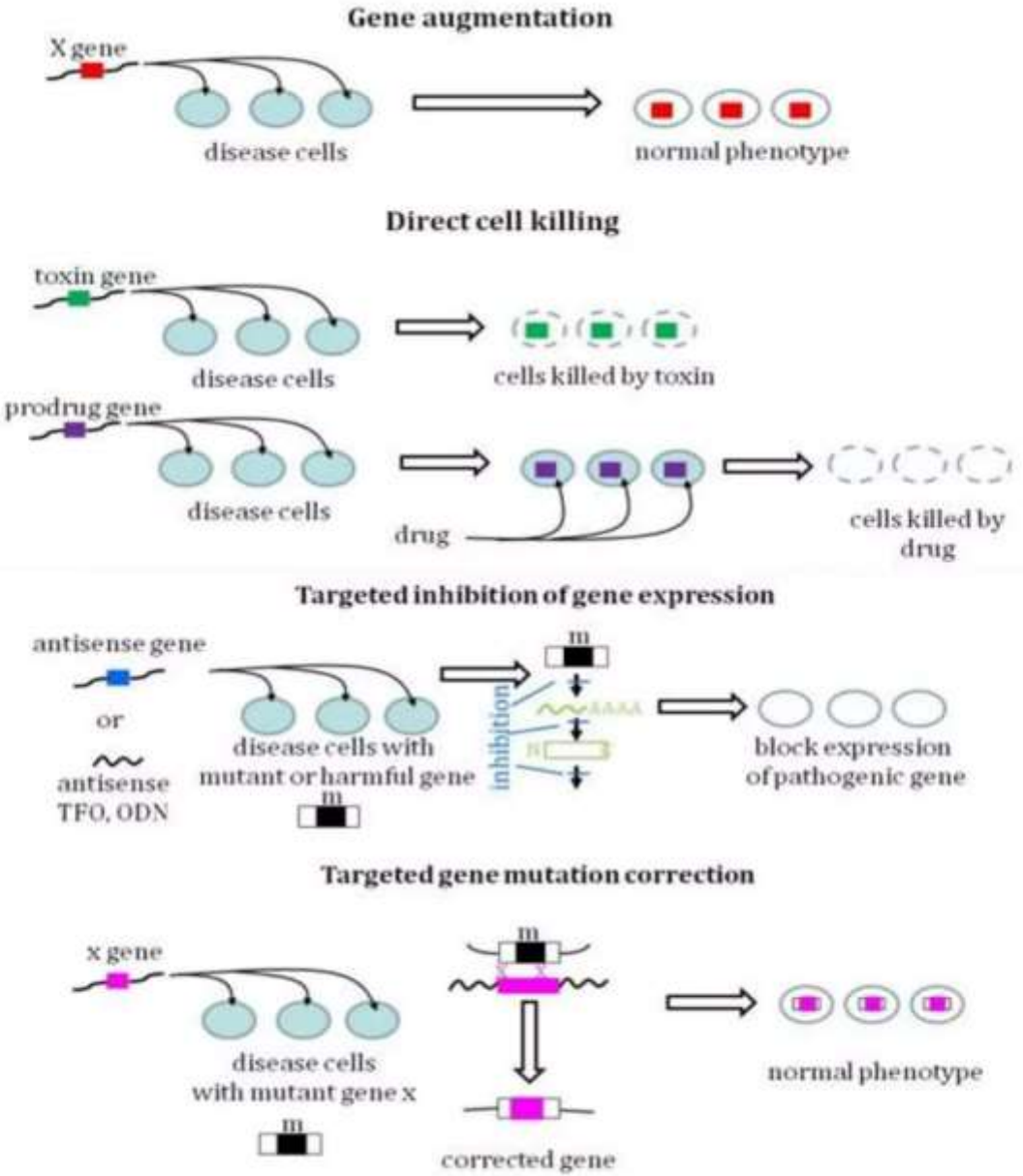
- The repair of a genetic defect to restore a functional allele.
- Technical difficulties have meant that it is not sufficient reliable to warrant clinical trails.
- In principle it can be done at different levels at the gene level or at the RNA transcript level.



TARGETED INHIBITION OF GENE EXPRESSION

- Suitable for treating infectious diseases and some cancers.
- The basics of this therapy is to eliminate the activity of agene that encourage the growth of disease related cells.
- If diseases cells display an inappropriate expression of a gene a variety of different system can be used specifically to block the expression of a single gene at the DNA, RNA or protein levels




Gene augmentation
X gene
disease cells
normal phenotype
Direct cell killing
toxin gene
disease cells
cells killed by toxin
prodrug gene
disease cells
drug
cells killed by drug
Targeted inhibition of gene expression
antisense gene
or
antisense TFO, ODN
disease cells with mutant or harmful gene
m
inhibition
block expression of pathogenic gene
Targeted gene mutation correction
x gene
disease cells with mutant gene x
m
corrected gene
normal phenotype

GENE TRANSFER TECHNIQUES

Non viral

Non viral systems comprises all the physical and chemical methods

Generally include chemical methods either chemical methods such as cationic liposomes and polymers or physical methods such as gene gun, electroporation, magnetofaction etc.

Viral vectors

Choice of viral vector is dependent on gene transfer efficiency, capacity to carry foreign genes, toxicity ,stability , immune response towards viral antigens and potential viral recombination.

RETROVIRUS VECTOR

- It is carried out by one of the enzymes carried in the virus, called reverse transcriptase. After this DNA copy is produced and is free in the nucleus of the host cell, it must be incorporated into the genome of the host cell.
- That is, it must be inserted into the large DNA molecules in the cell (the chromosomes). This process is done by another enzyme carried in the virus called integrase.
- One of the problems of gene therapy using retroviruses is that the integrase enzyme can insert the genetic material of the virus in any arbitrary position in the genome of the host. If genetic material happens to be inserted in the middle of one of the original genes of the host cell, this gene will be disrupted (insertional mutagenesis).
- If the gene happens to be one regulating cell division, uncontrolled cell division (i.e., cancer) can occur. This problem has recently begun to be addressed by utilizing:

  1- Zinc finger nucleases or

  2- by including certain sequences such as the beta-globin locus control region to direct the site of integration to specific chromosomal sites.
- Examples of diseases targeted using these viruses include: severe combined immunodeficiency (SCID) and leukemia.

ADENO VIRUS

- Adenoviruses are large linear double-stranded DNA viruses that are commonly used for preparing gene transfer vectors. Adenovirus vectors are known to be the second most popular gene delivery vector for gene therapy of various diseases like cystic fibrosis and certain types of cancer.
- It can rapidly infect a large range of human cells.
- Can hold large segments of DNA.
- DNA is easy to manipulate with current recombinant DNA tecniques.

ADENO-ASSOCIATED VIRUSES

- Small viruses with a genome of single stranded DNA. These viruses can insert genetic material at a specific site on chromosome 19.
- There are a few disadvantages to using AAV, including the small amount of DNA it can carry (low capacity) and the difficulty in producing it.
- Several trials with AAV are on-going or in preparation, mainly trying to treat muscle and eye diseases.
- Recent clinical trials have also been initiated where AAV vectors are used to deliver genes to the brain. This is possible because AAV viruses can infect non-dividing cells, such as neurons in which their genomes be expressed for a long time

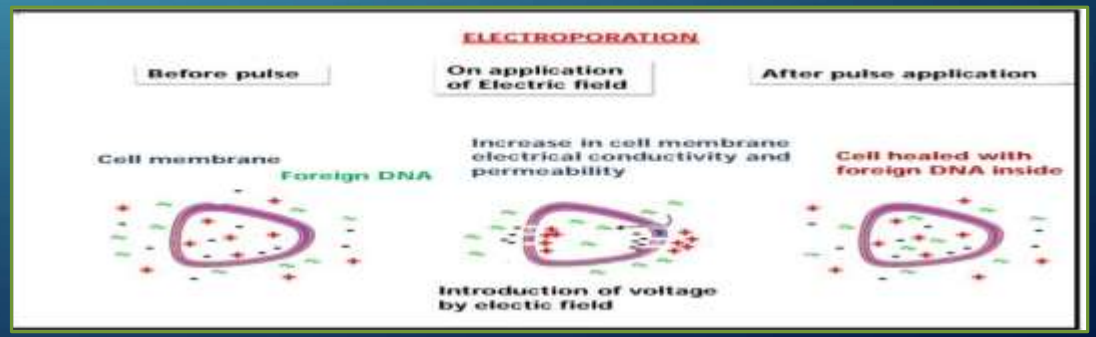
NON-VIRAL METHODS:

NACKED DNA

- This is the simplest method of non-viral transfection. Clinical trials have been carried out of intramuscular injection of a naked DNA plasmid have occurred with some success, however the expression has been very low in comparison to other methods of transfection.
- In addition to trials with plasmids, there have been trials with naked PCR product, which have had similar or greater success, however this success does not compare to that of the other methods, leading to research into more efficient methods for delivery of the naked DNA such as electroporation and the use of a "gene gun", which shoots DNA coated gold particles into the cell using high pressure gas.

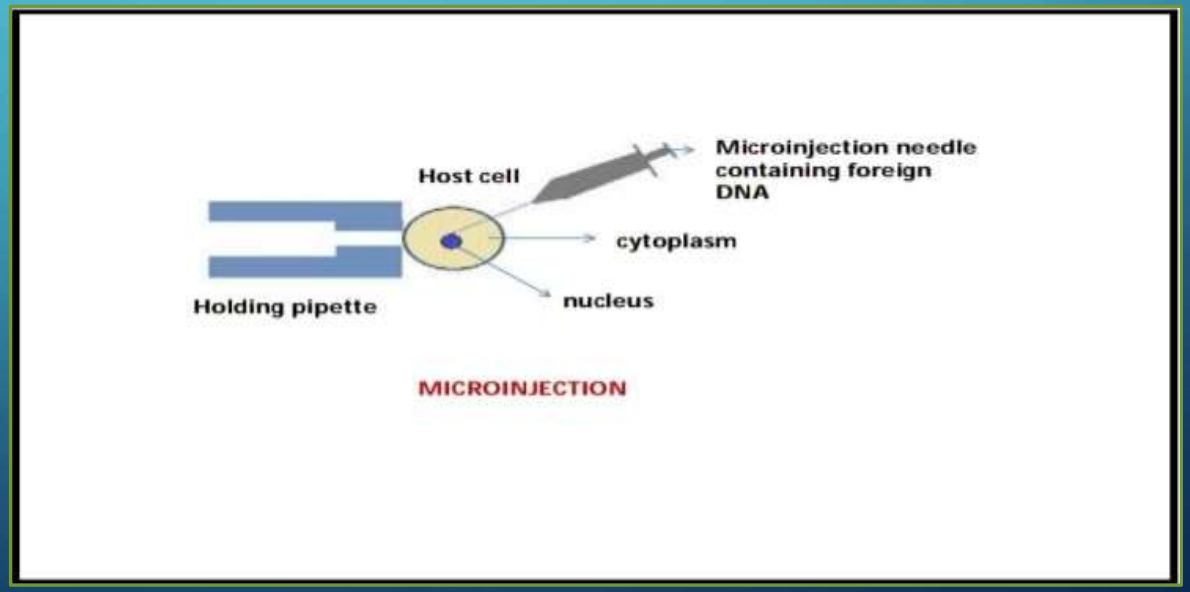
ELECTROPORATION

- In electroporation, the external electric field is applied to the protoplast, which changes the electrical conductivity and the permeability of cell membrane; and thus the exogenous molecules found in the medium are taken up to either the cytoplasm (transient transfection) or into the nucleus (stable transfection).



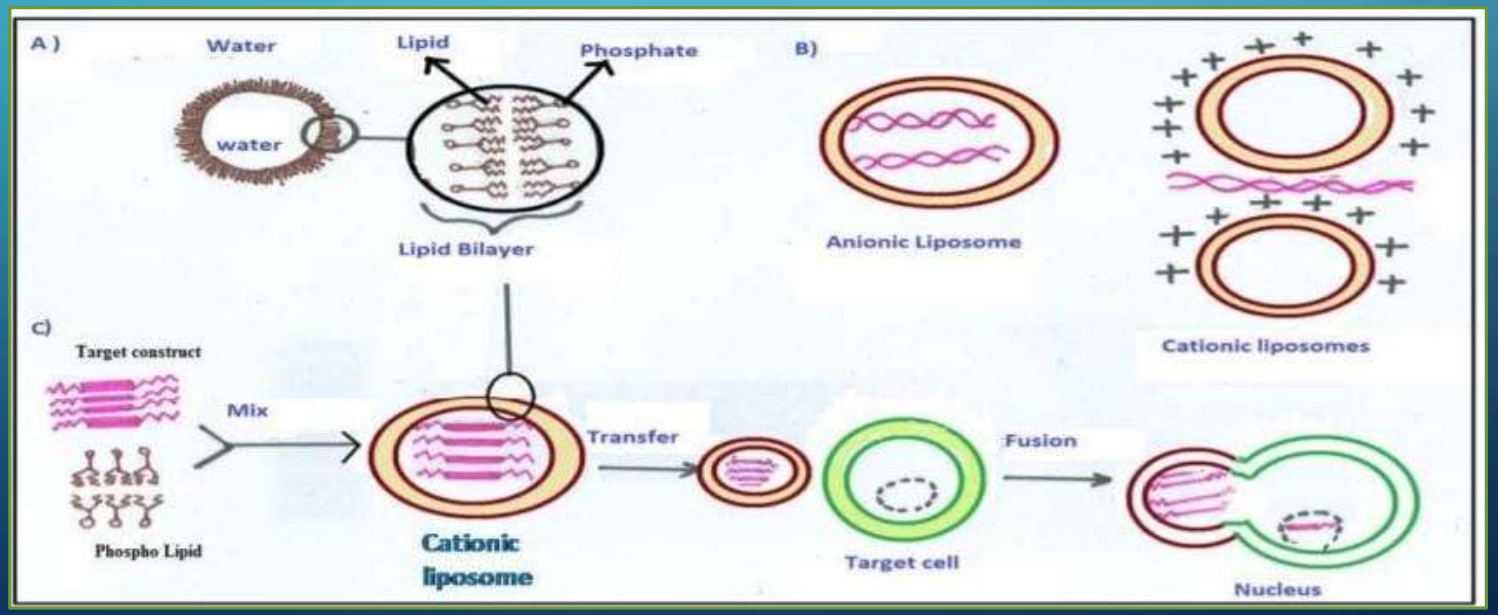
MICROINJECTION

- Microinjection involves the delivery of foreign DNA, by the help of glass micropipette into a living cell. The cell is held against a solid support or holding pipette and micro neeedle containing the desired DNA is inserted into the cell. The tip of the pipette used is about 0.5 to 5 micro meter diameter which resembles an injection needle. For this, glass micropipette is heated until the glass becomes somewhat liquefied and is quickly stretched to ressemble a injection needle. The delivery of foreign DNA is done under a powerful microscope (micromanupulator).



LIPOSOMES

- Liposomes are spherical vesicles which are made up of synthetic lipid bilayers which imitate the structure of biological membranes. DNA to be transferred is packaged into the liposome in vitro and transferred to to the targeted tissue. The lipid coating helps the DNA to survives in vivo and enters into the cell by endocytosis. Cationic liposomes, where the positive charge on liposomes is stabilized by binding of negatively charged DNA, are popular vehicles for gene transfer in vivo.



Advantages	Disadvantages
Provide greater gene transfer efficiency in both in vivo and in vitro environments	Can trigger severe immune responses and inflammatory reactions
Persist for longer periods of time in most cases	Their cloning capacity is very limited
Can target a large number of cells	Produced by complex production methods
A large variety of viruses are available to choose from	Low capability of tropism to some specific target cells
Innate ability of tropism toward infection	Can cause mutagenesis by inserting their exogenous DNA into the host genome
Capable of evading endosomes by various mechanisms learned by evolution of viruses	Research is needed to further understand the mechanisms of molecular infection by viruses

Table 1 Proof of principle for therapeutic *in utero* gene transfer (DG = days of gestation)

Animal model	*Vector*	*Route of application*	*Outcome*	*Ref.*
Gunn rat, model of Crigler Najjar disease Type I	HIV lentivirus expressing human UDP-glucuronyl-bilirubin transferase	Topical liver injection 17–19 DG	45% reduction of nonconjugated bilirubin for a period of 1 year	3
RPE65$^{-/-}$ mouse model of Leber's congenital amaurosis	AAV 2 expressing human RPE65	Subretinal injection 14 DG	54% of treated eyes showed improvement in ERG and 70% in photoresponse-sensitivity at 1–2.5 months after birth; RPE65 expression detectable up to 6 months	4
KO mouse model of GSDII deficiency (Pompe's disease)	AAV2 expressing acid α-glucosidase	Intraperitoneal injection on 15 DG	Restoration of normal GAA levels in diaphragm prevented glycogen accumulation and restored diaphragm contractility up to 6 months postpartum	5
Factor IX KO mice model for haemophilia B	HIV lentivirus expressing, human Factor IX	Yolk sac vessel injection on 15 DG	Permanent therapeutic levels of human factor IX at 18–32% of normal, lifetime phenotype correction of bleeding disorder and tolerance of human factor IX	6

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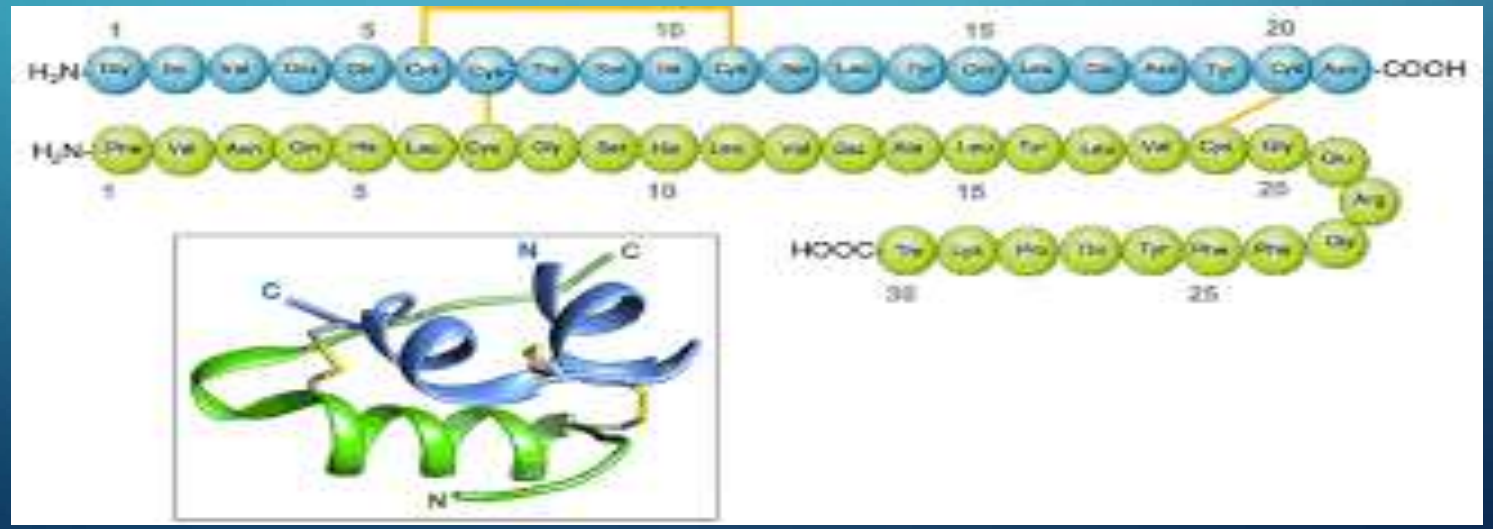
RECOMBINANT INSULIN PRODUCTION

INTRODUCTION

- The hormone insulin is produced by beta cells of islets of langerhans of Pancreas.
- Insulin is 51 aminoacid hormone, consisting of two polypeptide chains.(A chain & B chain).
- The polypeptide chains are held together by disulphide bonds.



METHODS OF INSULIN PRODUCTION

- Proinsulin method
- Two chain method

GENE ISOLATION

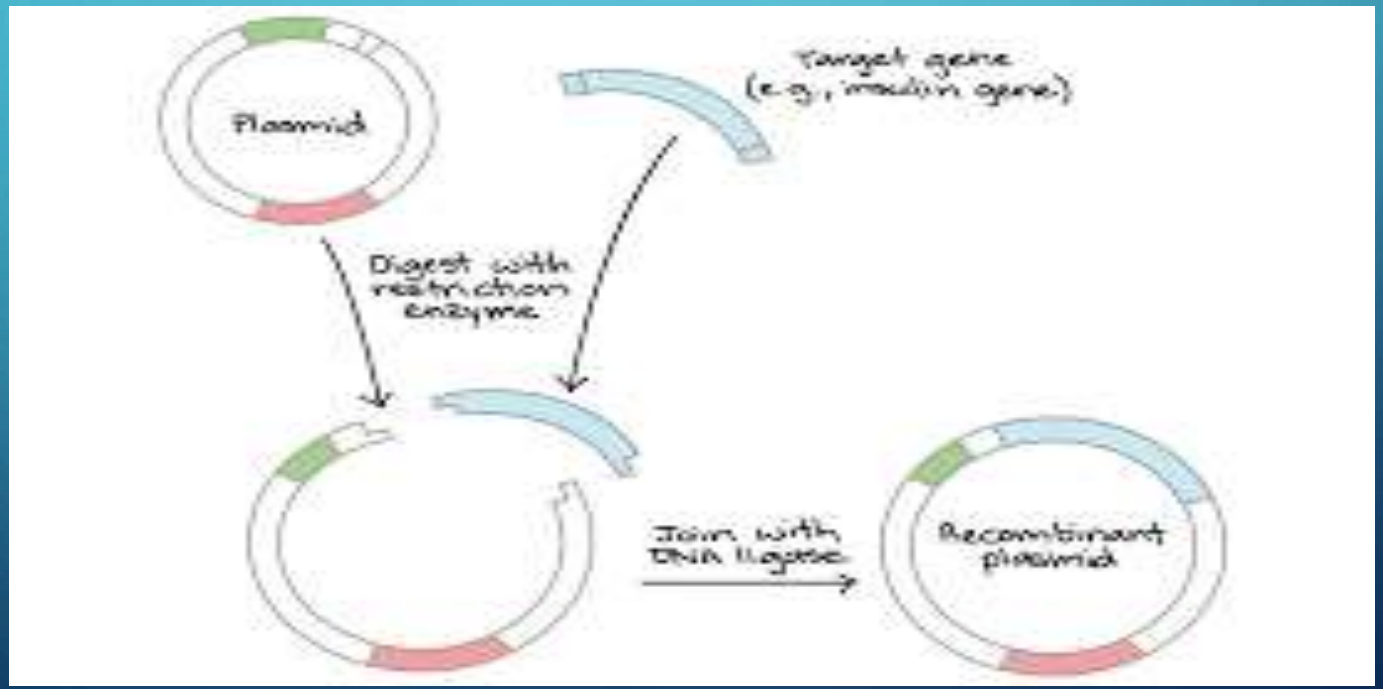
Complementary DNA (cDNA) molecules encoding chain A and chain B are obtained form human insulin mRNA using reverse transcription. The cDNAs of both chains are amplified by PCR.

INSERTION INTO THE PLASMID

- Plasmid is cut using restriction enzymes to insert the DNA sequence for the proinsulin producing gene.
- The restriction sites of EcoR1 and BamH1 contain one of the chain genes in plasmid.
- Specific DNA ligases are added to bind the inserted chain gene into the plasmid
- The entry of recombinant plasmids into bacterial cells is called transfection. Different techniques can be used for the transformation of *E. coli*, such as treatment with Calcium chloride or Electroporation techniues. After the plasmid's entry, the cells become transformed.

INSERTION INTO THE PLASMID

➢The transformed cells are then grown on tryptic soy broth containing the antibiotic kanamycin monosulfate resistance gene along with the proinsulin coding genes, so the transformed cells can survive in the broth.



FERMENTATION

- After 24 hours, the transformed E. coli has consumed and depleted all the nutrients in the test tubes, hence they are placed in the bioreactor to support further growth.
- This bioreactor has a total volume of 23L and a working volume of 16L. 1L of E. coli and depleted growth medium is taken and mixed with 9L of fresh growth medium in the bioreactor.
- Now, these cells will receive carbon from glycerol and yeast, nitrogen from ammonium sulfate and thiamine, and inorganic nutrients from potassium dihydrogen phosphate and dipotassium phosphate, which also act as buffers to maintain pH. Trace elements will be provided by sodium citrate, magnesium sulfate, and a vitamin solution.

FERMENTATION

➢The conditions within the bioreactor are monitored by biosensors. The E. coli reaches its maximum growth within 28 hours, after which it is removed from the surrounding medium through centrifugation.

CENTRIFUGATION

Cell isolation is the first step in down streaming of the insulin made by transformed E. Coli cells. Since E. Coli has the highest density of all the components in the growth medium, the bacterial cells settle to the bottom after centrifugation at 8185 rpm for 10 minutes.

HOMOGENIZATION

The bacteria and medium are injected into the chamber of a high-pressure homogenizer with intense speed, and as a result, when the mixture encounters the blade present within the chamber, a condition of high turbulence and shear is created leading to compression, acceleration, and a pressure drop

INCLUSION BODY SEPARATION BY CENTRIFUGATION

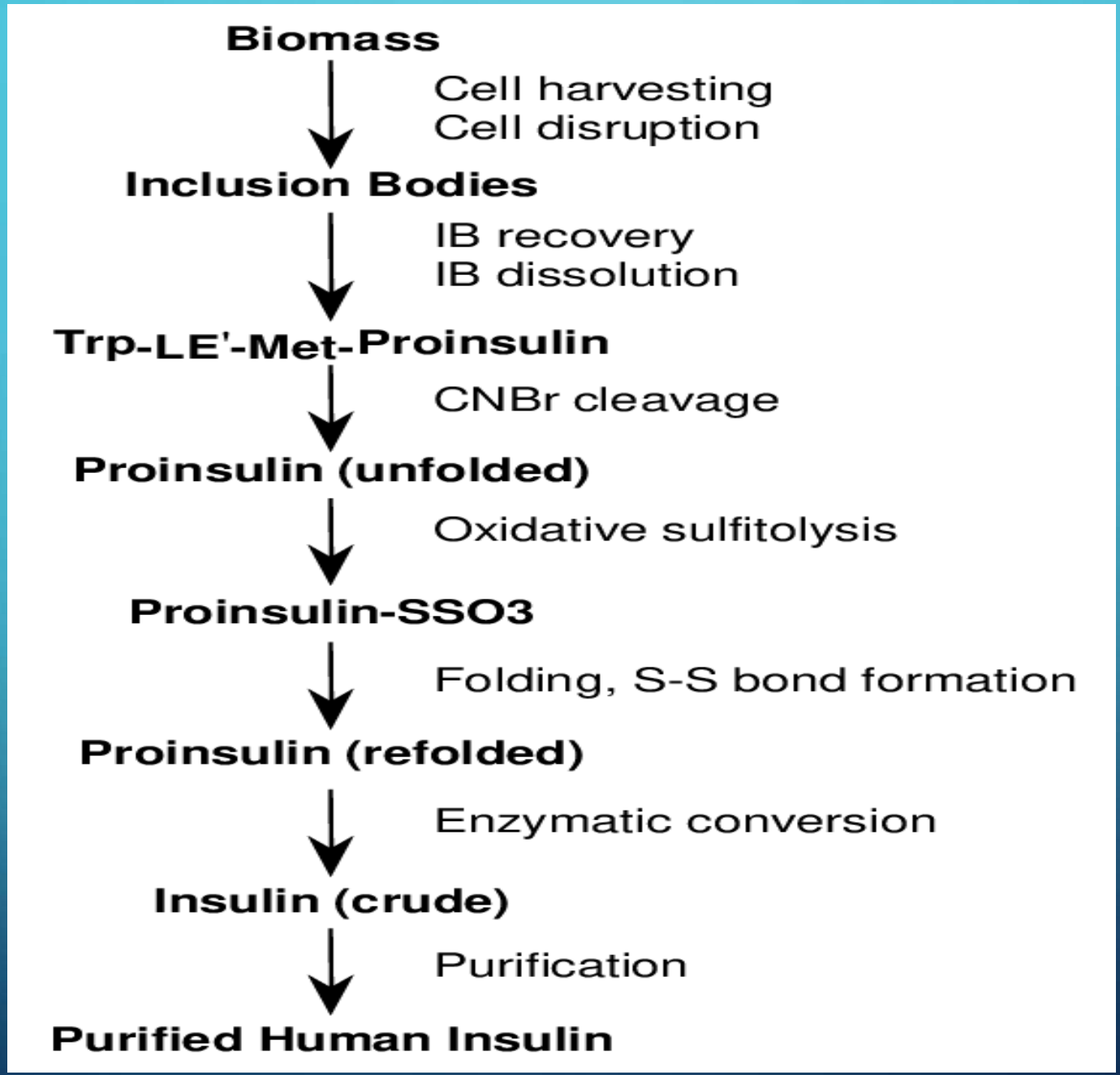
- After the E. coli have been lysed, the inclusion bodies need to be isolated from the cell debris. For this purpose, centrifugation can be used for reverse osmosis. Since the proinsulin inclusion bodies are dense, they will sink to the bottom.
- However, the speed of centrifugation must be higher (15000 x g for 30 minutes) than before, since the inclusion bodies have a lower density than the intact bacterial cells. After centrifugation, the supernatant is discarded while proinsulin and some impurities remain in the tube.

SOLUBILIZATION OF INCLUSION BODIES

- After the separation of inclusion bodies, proinsulin is in an insoluble form and therefore must be solubilized. This is accomplished through the addition of denaturing agents such as urea or guanidium hydrochloric acid, which will release the fusion proteins. This process is followed by the addition of either β-mercaptoethanol or DTT, which are reducing agents, to break the disulfide bonds present within the proinsulin fusion proteins.
- In the traditional proinsulin procedure, after solubilization, a cleavage step for the preparation of proinsulin is performed. This step can also be performed later in the processing. It involves adding cyanogen bromide and 70% formic acid to cleave the peptide linker between proinsulin and its fusion protein partner.

SULFITOLYSIS

- Sulfitolysis first involves breaking the disulfide bonds by adding reducing agents. These bonds get broken during solubilization or other earlier steps of purification.
- The process of sulfitolysis is performed along with 6 hours of solubilization, and 0.8M Na_2SO_4 and 0.3M $Na_2SO_4*H_2O$ are added to facilitate oxidation and maintain the unfolded form of proinsulin. Sulfite (SO_3) ions are added to cysteine molecules, which prevents the formation of incorrect disulfide bonds.
- All of the cysteine residues in the proinsulin molecule have sulfite ions added. However, the ZZ tail has no cysteine residues, therefore it remains unaltered and is utilized in further downstream processes.


Biomass
Cell harvesting
Cell disruption
Inclusion Bodies
IB recovery
IB dissolution
Trp-LE'-Met-Proinsulin
CNBr cleavage
Proinsulin (unfolded)
Oxidative sulfitolysis
Proinsulin-SSO3
Folding, S-S bond formation
Proinsulin (refolded)
Enzymatic conversion
Insulin (crude)
Purification
Purified Human Insulin

DIALYSIS

This process is used to remove the previously used denaturants and dissolved reagents without chemically modifying the fusion protein product. It involves the addition of buffers such as 10mM Tris-HCl (4 repetitions) to remove reagents including urea, DTT, and β-mercaptoethanol, and initiate the refolding process of the proinsulin fusion protein.

RENATURATION

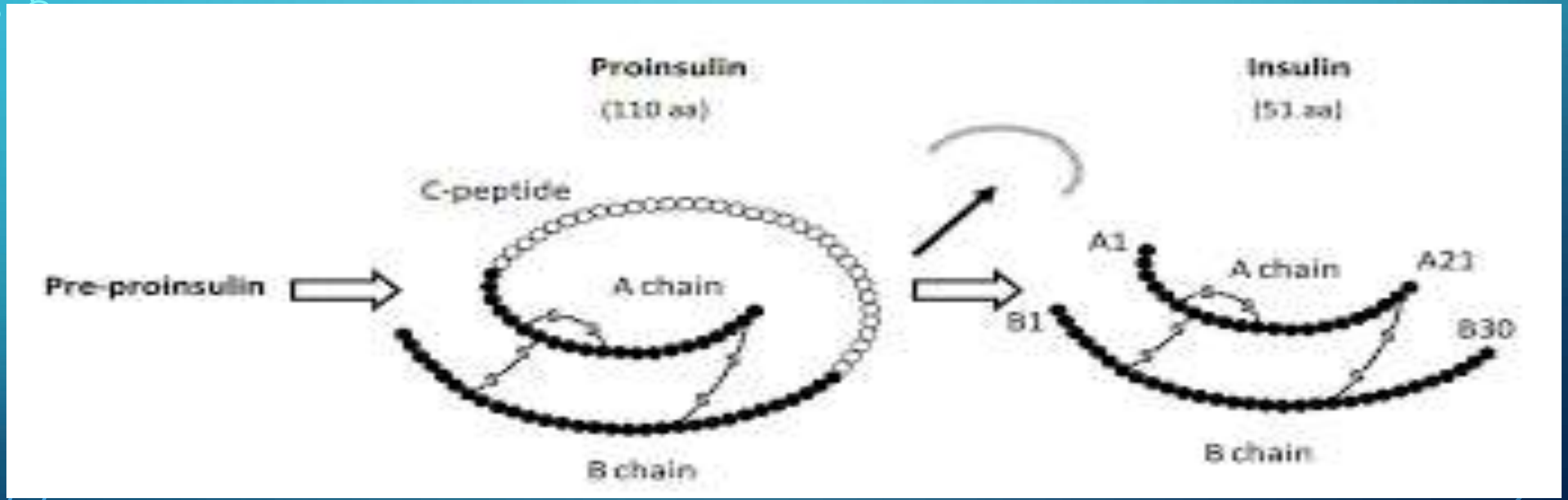
- The process of renaturation involves the correct folding of proteins, which depends heavily upon the correct formation of disulfide bonds.
- Renaturation is carried out for 20 hours at 4°C with the addition of 1M glycine-sodium hydroxide buffer (pH 10.5 or higher) and β-mercaptoethanol at an 18:1 molal ratio to the fusion protein.
- There are several methods available. Two commonly used methods are (1) the use of oxidative buffers such as low molarity Tris-HCl or glycine-sodium hydroxide to oxidize the reduced proinsulin, or (2) conversion of the proinsulin to the S-hexa-sulfonated form via sulfothiolysis using sodium sulfite, followed by the addition of redox reagents such as cysteamine, GSH or cysteine couples

AFFINITY CHROMATOGRAPHY

The results of affinity chromatography are observed using SDS-PAGE on a homogenous 12% gel. It involves the use of size exclusion chromatography with a Superdex 75 PC3.2/10 column with the addition of 200 mM sodium phosphate buffer at a flow rate of 100ml/min. ZZ-R proinsulin is resistant to degradation by proteases when passed through affinity chromatography. It is then further purified by size exclusion chromatography, whose recovery rate is 70%. Most of the recovered proinsulin is a monomer, while other forms of ZZ-R proinsulin are also present in small quantities

SITE SPECIFIC CLEAVAGE

After IgG chromatography, the purified ZZ-R proinsulin undergoes ultra-filtration which reduces its volume by 5 times, increasing its concentration to 12ml/min. Ultra-filtration is followed by cleavage of proinsulin into the C-peptide and insulin using trypsin and carboxypeptidase B. Trypsin is used for the breakdown of protein in the digestive system while carboxypeptidase is used for cleavage of proinsulin at a specific site to convert proinsulin into native insulin and the C-peptide..


Proinsulin
(110 aa)
Insulin
(51 aa)
C-peptide
Pre-proinsulin
A chain
A1
A chain
A21
B1
B30
B chain
B chain

REVERSE-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

➢Reverse-phase high-performance liquid chromatography (RP-HPLC) is used to separate the C-peptide and human insulin. RP-HPLC is a common method used to analyze insulin products since it can separate insulin into its different species, and the use of high pressure increases the speed of the process and purity of the product. RP-HPLC involves a non-polar stationary phase and a polar mobile phase. Since insulin is non-polar and large, it adheres to the stationary phase column whereas the mobile phase contains methanol or acetonitrile in a buffer solution to analyze the insulin.

POLISHING WITH ZINC COMPLEXATION

- Eli Lilly and Co. have devised a method that requires only daily 2 insulin injections for people with an active lifestyle. They used zinc ions to form crystal complexes with insulin, named 2Zn insulin, which have a hexagonal configuration while exhibiting axial symmetry, hence slowing the release of insulin to the body and preventing its immediate use by cells.
- Cobalt can also be used for this purpose. These metallic ions form weak ionic bonds with insulin, causing insulin molecules to congregate and form suspended crystals. The formation of crystals is achieved through batch crystallization, and the solution is stored at 4°C. The better and more regular the crystal, the longer it can remain active in the bloodstream

INSULIN

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