ВОЛГОГРАДСКИЙ ГОСУДАРСТВЕННЫЙ МЕДИЦИНСКИЙ УНИВЕРСИТЕТ

Vectors as carriers for gene delivery. Main types and principles of vector action.

The main stages of molecular biology.

- The first romantic period 1935-1944. Max Delbrück and Salvador Luria studied the reproduction of phages and viruses, which are complexes of nucleic acids with proteins.
- In 1940 George Beadle and Edward Tatum formulated the hypothesis - ‘One gene - one enzyme’. However, what a gene is in physico-chemical terms was not yet known at that time.
- Second romantic period 1944-1953 The genetic role of DNA was proved.
- In 1953, the model of the DNA double helix appeared, for which its creators James Watson, Francis Crick and Maurice Wilkins were awarded the Nobel Prize.
- Dogmatic period 1953-1962. The central dogma of molecular biology is formulated: The transfer of genetic information proceeds in the directionDNA → RNA → protein.
- In 1962, the genetic code was decoded. The academic period from 1962 to the present, in which the genetically engineered sub-period has been distinguished since 1974.

Major discoveries in molecular biology:

- 1928 - F. Griffith discovered the phenomenon of transformation in Streptococcus pneumoniae.
- 1941 - D. Beadle and E. Tatum put forward the statement ‘One gene - one enzyme’ on the basis of the study of axotrophic mutants of Neurospora crassa.
- 1944 - O. Avery, C. Mac Leod and M. Mac Carthy show in an experiment that the transforming factor discovered by F. Griffith is represented by DNA, not protein.
- 1952 - Experiment by A. Hershey and M. Chase in which the hereditary role of DNA is proved.
- 1953 - J. Watson and F. Crick described the structure of DNA based on the results of X-ray structural analysis obtained by R. Franklin and M. Wilkins.
- 1956 - A. Kornberg isolated DNA polymerase I from E. coli cells.
- 1957 - F. Crick formulates the central dogma of molecular biology.
- 1960 - A. Pardet, F. Jacob, and J. Monod show the necessity of RNA (iRNA) synthesis for gene expression.
- 1960 - 1964. - Experiments on deciphering the genetic code.
- 1970 - H. Tyomin and D. Baltimore independently discover the reverse transcriptase of retroviruses.

The main stages of molecular biology.

- Late 60‘s - early 70’s - S. Linn, V. Arber, D. Nathans and G. Smith conduct a study of the retorting enzymes of E. coli and H. influenzae bacteria.
- 1972 - A full-length tRNA gene is synthesised from single nucleotides in the laboratory of H. Gobind Korana.
- 1972 - P. Berg was the first to create a recombinant DNA molecule by restriction and ligation of DNA fragments.
- 1973 - S. Cohen and G. Boyer, using the restriction-ligase method, obtained recombinant plasmids and transformed Escherichia coli cells with them.
- 1976-1979. - Commercial strains of E. coli containing genes for insulin, somatostatin and human growth hormone were obtained.
- 1977 - F. Sanger developed a method of DNA sequencing based on the use of termination nucleotides.
- 1981-1982 - mice with foreign genes of β-globin and growth hormone, as well as the first transgenic plants (tobacco with antibiotic resistance genes of bacteria) were produced.
- 1983 - C. Mullis performed the first polymerase chain reaction (PCR).
- 1994 - recombinant green fluorescent protein (GFP) of the jellyfish Aequorea victoria.
- 2003 - completion of a thirteen-year international project to sequence the human genome.
- Late 2000s - emergence of new methods of recombinant DNA production that do not require restriction and ligation.
- 2010 г. - Creation of the first Mycoplasma mycoides bacteria with an artificial (chemically synthesised) genome.
- 2010s - widespread development of in vivo genomic DNA editing methods.

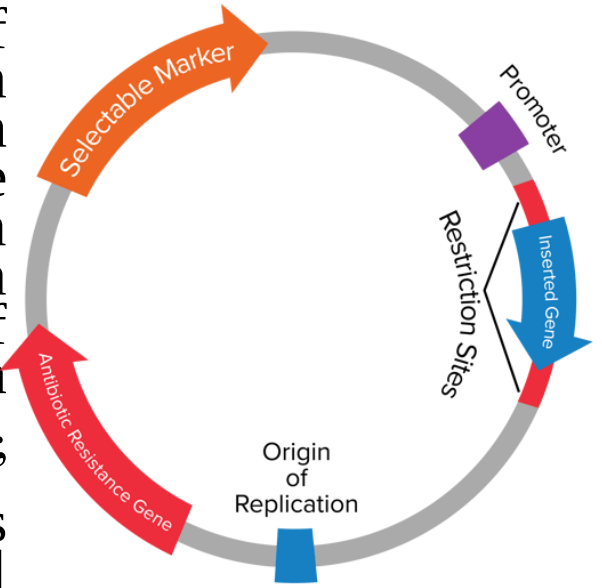
The concept of vector. Characterisation of the main genetic elements of pro- and eukaryotic cells claiming to be vectors.

Vector is a nucleic acid molecule, most often DNA, used in genetic engineering for transferring genetic material inside a cell, including into a cell of a living multicellular organism in vivo. Two main vectors are used to introduce recombinant DNA: plasmids, bacteriophages.

Plasmid vectors

Plasmids are extrachromosomal genetic elements in the form of circular DNA molecules containing 1-3% of the genome of a bacterial cell. All bacteria have plasmids. Some of them contain information that ensures their own transfer from one cell to another (F-plasmids), others carry antibiotic resistance genes (R-plasmids) or specific sets of genes responsible for the utilisation of metabolites (degradation plasmids). Each plasmid contains a replication start site, without which replication of the plasmid in the host cell is impossible. If two or more plasmids cannot coexist in the same cell, they belong to the same incompatibility group. Plasmids belonging to different incompatibility groups exist unhindered in one cell regardless of the number of copies. In some microorganisms, up to 10 different plasmids have been found in one cell, each of which performed different functions and belonged to a different incompatibility group. Replication of plasmids proceeds independently of chromosome replication. The number of copies is determined by the regulatory system of the cell. A bacterial clone containing such a plasmid can be compared to a factory for the production of this fragment.

It is fundamentally important that vectors (in particular, plasmid vectors) have properties characteristic of them: 1. replication initiation point (ori) - the sequence of nucleotides from which DNA doubling begins. If vector DNA cannot double (replicate), the necessary therapeutic effect will not be achieved, because it will simply be rapidly cleaved by intracellular enzymes-nucleases, and due to the lack of matrices, much fewer protein molecules will be formed in the end. It should be noted that these points are species-specific, i.e. if vector DNA is to be produced by replicating it in bacterial culture (rather than simply by chemical synthesis, which is usually much more expensive), then two replication start points will be required separately, one for humans and one for bacteria; 2. Restriction sites - specific short sequences (more often palindromic) that are recognised by special enzymes (restriction endonucleases) and cut by them in a certain way - with the formation of ‘sticky ends’. These sites are necessary to stitch the vector DNA (which, in fact, is a ‘dummy’) with the desired therapeutic genes into a single molecule. Such a molecule sewn from two or more parts is called a ‘recombinant’ molecule; 3. Clearly, it would be desirable for us to obtain millions of copies of a recombinant DNA molecule. Again, if we are dealing with a bacterial cell culture, then further this DNA needs to be isolated. The problem is that not all bacteria will swallow the molecule we need, some will not. In order to distinguish between these two groups, selective markers are inserted into the vector DNA, i.e. areas of resistance to certain chemical substances; now, if we add these substances to the medium, only those that are resistant to them will survive, and the rest will die.



Plasmid vectors

Plasmid vectors are usually created by genetic engineering, as natural (unmodified) plasmids lack a number of properties required for a ‘high-quality vector’:

small size, as the efficiency of exogenous DNA transfer into E. coli decreases when the length of the plasmid exceeds 15 thousand nucleotide pairs;

the presence of a restriction site into which the insertion is made;

presence of one or more selective genetic markers for identification of recipient cells carrying recombinant DNA.

Plasmids are inserted into somatic cells in a variety of ways. The frequency of transformation is not 100%, then use selection schemes to identify transformed cells. As markers, the plasmid may contain genes that determine the antibiotic resistance of the bacterium. Insertion of a foreign (donor) gene into the marker gene leads to inactivation of the latter. This makes it possible to distinguish transformed cells that have received a vector plasmid (that have lost resistance to an antibiotic) from cells that have received a recombinant molecule (that have retained resistance to one antibiotic but lost resistance to another). This technique is called inactivation of the insertion marker. To select transformed cells containing recombinant DNA (hybrid plasmid), resistance to certain antibiotics is tested. For example, cells carrying the hybrid plasmid are resistant to ampicillin, but sensitive to tetracycline (in the marker gene of which the donor DNA is inserted). The process of dividing genomic DNA into clonable elements and introducing these elements into host cells is called the creation of a genomic library (clone bank, gene bank). All cloning systems must fulfil two basic requirements. The availability of multiple sites for cloning. The ability to easily identify cells with recombinant DNA.

Typically, the following characteristics guide the selection or design of a suitable viral vector:

1. Capacity - the length of DNA of the target gene that can be placed in the vector.
2. Selectivity of uptake by cells targeted for a given therapy and lack of expression in tissues where the resulting protein may cause toxicity (e.g., heart).
3. Duration of gene expression.
4. Immunogenicity - the effect of the vector on the immune response.
5. Ease of production.
6. Ability to integrate into the cell DNA or ability to exist as a stable element in the cell nucleus without genomic integration.
7. The likelihood of the patient having antibodies against the vector if the organism has previously encountered a similar virus - this reduces the effectiveness of the vector.

Thus, an ideal vector must possess:

- with places for easy insertion of DNA fragments;
- sufficient capacity;
- selective markers to identify cells with this vector, both ‘empty’ and ‘inserted’;
- DNA regions that ensure its maintenance as a separate replicon or integration of the cloned fragment into the host genome;
- DNA regions providing (if required) efficient expression of the inserted gene in the selected host.

For all routine molecular cloning procedures, E. coli is widely used as a host cell.

Cells capable of absorbing foreign DNA are called competent cells; E. coli competence is enhanced by using special cultivation conditions. To obtain large amounts of foreign proteins using recombinant E. coli strains, a plasmid containing a strong promoter, a selective marker gene and a short region with several unique sites for restriction enzymes - a polylinker - was designed. Effective methods of transformation of E. coli with plasmids are electroporation (exposure of cell membranes to an electric current to increase their permeability). To introduce cloned genes into somatic cells, microinjections, micro-pricking or fusion of DNA-laden membrane vesicles (liposomes) with the cell are also used. Despite the current predominance of virus-based vectors, genes can also be delivered by other physical and chemical methods: gene gun, magnetofection, sonoporation, application of various nanoparticles (made of silicon, gold, calcium phosphate, lipids), and others.

Physical methods of delivery of genetic material.

The classical physical method of genetic material delivery is the so-called '**gene gun**', first used for gene transfer in plants. The method is based on bombarding cells or tissue with metal particles coated with DNA. The necessary speed is imparted to the particles by the current of carrier gas (most often helium) or by high-voltage electric discharge. Gold, tungsten or silver microparticles with a diameter of about 1 μm are used as a carrier. To achieve the necessary efficiency of genetic material transfer and reproducibility of results, accurate standardisation of all physical and technical parameters of the experiment (particle size, gas pressure, etc.) is necessary. Currently, gene guns are used in ovarian cancer research. An obvious disadvantage of gene guns is the traumatisation of target tissues, often resulting in the death of transfected cells.

Method of electroporation

Electric discharges can also increase the permeability of the plasma membrane. This is the basis of the **electroporation method**, another classical physical method of gene transfer. Application to the plasma membrane of an electric field larger than its own electrical capacitance causes redistribution of charges on the membrane with subsequent formation of pores, which allows DNA molecules to diffuse inside the cells. Field parameters are selected based on the physical properties of the target cell membrane. **Intradermal**, **intramuscular** and **intratumoural** delivery of plasmid DNA by electroporation has been described. The main obstacle to the use of electroporation in vivo is also the increased death of cells exposed to the electric field.

The sonoporation method

A more gentle technique of plasma membrane permeabilisation is ***sonoporation*** - ultrasound treatment of the target tissue. To deliver genetic material into cells by sonoporation, DNA is immobilised on the surface of microbubbles and injected into the bloodstream with subsequent application of ultrasound in the projection of the target organ. The bubbles consist of a core (perfluorocarbons or sulphur hexafluoride) filled with gas (air, nitrogen, inert gas) and coated with lipids, proteins or synthetic biopolymers. Circulating microbubbles, which are similar in size to red blood cells (diameter 2-4 μm), respond to ultrasound and release DNA, which diffuses into the permeabilised cells.

Sonoporation is used to deliver genetic material to the brain, kidneys, abdominal cavity, and muscle tissue, including cardiac muscle, among others.

«Cell squeezing»

«Cell squeezing» – is a method invented in 2013. It allows molecules to be delivered into cells by ‘gently squeezing’ the cell membrane. The method eliminates the possibility of toxicity or mis-targeting because it does not depend on external materials or electric fields.

The hydroporation method

In rare cases, hydroporation is used to deliver genetic material in vivo, i.e. increasing membrane permeability due to a sudden change in hydrodynamic pressure. The pressure is created by injection of large volumes of DNA solutions in a short period of time. This effect increases the permeability of capillary endothelium and forms pores in the plasma membrane of surrounding parenchyma cells through which DNA penetrates. This method is usually used for gene therapy of liver cells.

DNA microinjection

DNA microinjection - introduction into the cell nucleus using thin glass microtubules (d=0.1-0.5 μm).

Disadvantage - complexity of the method,

- high probability of destruction of the nucleus or DNA;
- a limited number of cells can be transformed;
- is not used for humans.

Particle-based methods.

A direct approach to transfection is the gene gun, in which DNA is bound into a nanoparticle with inert solid substances (more often gold, tungsten), which is then ‘shot’ in a directed manner into the nuclei of target cells. This method is used in vitro and in vivo to introduce genes, in particular, into muscle tissue cells, for example, in such a disease as Duchenne myodystrophy. The size of gold particles is 1-3 microns.

Particle-based methods.

A very effective method for DNA transfection is to introduce it through liposomes, which are small membrane-encircled cells that can fuse with the cell cytoplasmic membrane (CPM), which is a double layer of lipids. For eukaryotic cells, transfection is done more efficiently using cationic liposomes because the cells are more sensitive to them. The process has its own name - lipofection. This method is now considered one of the safest. Liposomes are non-toxic and non-immunogenic. However, the efficiency of gene transfer using liposomes is limited because the DNA deposited by them in cells is usually immediately taken up by lysosomes and destroyed.

Particle-based methods.

Another method is the use of cationic polymers such as diethylaminoethyl dextran (DEAE-dextran) or polyethylenimine. Negatively charged DNA molecules bind to positively charged polycations and this complex further penetrates the cell by endocytosis. DEAE-dextran alters the physical properties of the plasma membrane and stimulates the uptake of this complex by the cell. The main disadvantage of the method is that DEAE-dextran in high concentrations is toxic. The method is not widespread in gene therapy.

Delivery by histones and other nuclear proteins

Histones are a broad class of nuclear proteins that fulfil two main functions: they participate in the packaging of DNA strands in the nucleus and in the epigenetic regulation of such nuclear processes as transcription, replication and repair.These proteins, which contain many positively charged amino acids (Lys, Arg), in vivo help to compactly pack a long DNA strand into a relatively small cell nucleus.

Magnetofection

Magnetofection is a technique that uses the forces of magnetism to deliver DNA into target cells. First, nucleic acids are associated with magnetic nanoparticles and then, under the influence of a magnetic field, the particles are driven into the cell. The efficiency is almost 100% and apparent non-toxicity has been observed. Already after 10-15 min the particles are registered in the cell - this is much faster than other methods.

Impalefaction

Impalefection (impalefection; 'impalement', lit. 'impalement' + 'infection') is a delivery method using nanomaterials such as carbon nanotubes and nanofibres. In this process, cells are literally pierced by a bed of nanofibrils. The prefix 'nano' is used to denote their very small size (within billionths of a metre).

Naked RNA и DNA

In addition, the possibility of delivering naked nucleic acids (naked RNA and DNA) is also being investigated. Such methods of delivery of target genes may theoretically have advantages over viruses, since their use is easier to mass produce (vectors based on nanoparticles, for example, are much easier to produce on an industrial scale), besides, the risk of genotoxicity and immunogenicity is also potentially low. However, so far, chemical gene delivery methods are less specific and precise than viral delivery and therefore less effective, and methods of physical introduction of genes into cells cannot be used for in vivo therapy. So far, no drugs based on non-viral gene delivery methods have been approved for use in humans.

Vectors based on bacteriophages

The use of bacteriophages as carriers of genetic information is based on the fact that the recombinant gene is incorporated into the virus genome and subsequently replicates with the virus genes during multiplication in the infected host cell. For this purpose, bacteriophage λ, a virus with double-stranded DNA that binds into a ring after entering the cell, is used. Bacteriophage M-13 is a filamentous virus with circular closed DNA, which turns into double-stranded DNA in the cell and replicates in progeny cells.

Vectors based on bacteriophages

In search of eukaryotic expression systems to produce biologically active proteins, bacmids - expression vectors based on baculoviruses for E. coli and insect cells - were created. The yield of recombinant baculoviruses in such a system increased up to 99%. Insect cells infected with baculoviruses synthesised a heterologous protein. Phage-based vectors are convenient for creating clonets (gene banks), but not for fine manipulations with a DNA fragment. For detailed study and transformation, DNA fragments are recloned into plasmids. In addition to the above vectors, cosmids are used in genetic engineering, i.e. plasmids carrying a cos-partition (complementary sticky ends) of phage λ DNA. The presence of the cos-part allows DNA packaging into the phage head in vitro, which ensures the possibility of their introduction into the cell by infection rather than transformation. Phasmids, hybrids between phages and plasmids, are capable of evolving as both a phage and a plasmid. Inferior to cosmids in terms of cloning capacity, phasmids make it possible to dispense with the recloning of genes from phage, into plasmid vectors. Thus, to obtain any protein product, it is necessary to ensure proper transcription of the gene encoding it and translation of the corresponding mRNA. A promoter is required to initiate transcription (RNA synthesis) at the desired site, and a terminating codon is required to stop it. The common yeast S. cerevisiae is intensively used for the synthesis of a variety of proteins encoded by cloned genes; the genetics of these single-celled organisms is well studied. Recombinant proteins synthesised in S. cerevisiae expression systems are used as vaccines and drugs. It is possible to give new properties to existing proteins and create unique enzymes by making specific changes using plasmids or PCR. Cloned genes make it possible to produce proteins containing the desired amino acids in the specified sites. Making specific changes to DNA coding sequences that result in specific changes in amino acid sequences is called directed mutagenesis

Disadvantages of gene therapy

Gene therapy technologies are at the very beginning of their journey, so they have a huge room for improvement. The following are the main challenges that need to be addressed:

- complexity, labour-intensive, expensive and, as a consequence, poor scalability of the technology, because of which gene therapies are still incredibly far from mass availability (and it is not certain that they will approach it any time soon);
- the exorbitant cost of such treatments, which means that they are only available to a few people;
- often serious adverse events of a new type, sometimes even leading to irreversible consequences (up to lethal outcome). However, with the accumulation of experience, doctors are already learning to cope with them;
- insufficient period of observation over the use of gene therapy, hence the uncertainty about its long-term effect, as well as concerns about the possible long-term consequences of using such drugs;
- imperfect technology of targeted gene delivery: viral vectors, which are most often used, are far from ideal.

In addition to the mentioned problems with non-targeted integration into the genome (typical for lentiviral and γ-retroviral vectors), there is also a danger of immune system activation (for AAV vectors), which reduces the efficiency of target gene delivery. In this case, reintroduction of the vector is not always possible.