HUMAN GENETIC DISORDERS

PART 2 – DIAGNOSIS AND TREATMENT

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1.1. Introduction

The first genetic test was run in 1978 by Kan and Dozy and independently by Orkin et al. The molecular testing was performed to detect the mutation c.20A>T in the β-globin DNA responsible for sickle cell anaemia. Kan and Dozy applied restriction analysis of the β-globin gene followed by hybridisation with a molecular probe complementary to the fragment of human β-globin sequence (RFLP method) [1]. Results enabled them the discrimination between mutant homozygotes (affected persons), normal homozygotes (healthy persons) and heterozygotes (mutation carriers). To detect the same mutation, Orkin et al. applied hybridisation with two variant oligonucleotide molecular probes to distinguish between mutant and normal alleles of the β-globin gene [2]. A milestone in development of molecular diagnostic techniques for detection of human mutations was the invention of polymerase chain reaction (PCR) made in 1983 by Kary Mullis. The PCR in molecular diagnostics is generally used to amplify DNA fragment before other molecular analyses are performed.

Genetic testing is divided in two categories: direct testing and indirect testing (gene tracking). **Direct genetic testing** involves testing for a certain genotype, i.e. identification of a specific pathogenic mutation within a gene, that is responsible for a certain condition. **Indirect genetic testing** also known as gene tracking tests the inheritance and segregation of genetic markers linked to a specific condition within a family.

1.2. Nomenclature of mutations

To describe sequence variations in a specific gene, a reference sequence should be used and its accession number from a database, such as Genebank, EMBL etc., should be specified in a publication.

Mutations at DNA level are preceded with letters denoting the type of reference sequence used to describe the mutation, i.e. description of a mutation is preceded with "**g.**" if genomic DNA is used as a reference sequence or with "**c.**" if cDNA is used as reference sequence or with "**m.**" if mitochondrial DNA is used as reference sequence. Sequence changes are described in upper case letters using A (adenine), C (cytosine), G (guanine) and T (thymine) to describe bases. Numbering of nucleotides begins with nucleotide +1 which is the A of the ATG codon (translation initiation codon). The previous nucleotide to the +1 nucleotide is described as -1 nucleotide (there is no 0 nucleotide). In case of intronic sequences, for the beginning of an intron, the description begins with the number of last nucleotide of the preceding exon, the "+" sign and the position in the intron (e.g. c.78+1G). For the end of an intron the description begins with the number of the first nucleotide of the

following exon, the "-" sign and the position located in the intron (e.g. c.79-1A). See Table 1 for general recommendations for description of mutations at DNA levels.

Table 1. General recommendations for description of mutations at DNA level. Based on ref. [3]. Depending on the type of reference sequence the description must be preceded with "g.", "c." or "m."

Type of change	**Sign**	**Example**	**Description**
Substitution	>	38A>C	Nucleotide 38 is changed from A to C
Deletion	del	38_40del (or 38_40delAGT)	Nucleotides from 38 to 40 (which are AGT) are deleted
Duplication	dup	38_40dup (or 38_40dupAGT)	Nucleotides from 38 to 40 (which are AGT) are duplicated
Insertion	ins	38_39insT	Nucleotide T is inserted between nucleotides 38 and 39
Two changes in one allele	[;]	[38A>C; 77_79del]	In the same allele, nucleotide 38 is changed from A to C and three nucleotides from 77 to 79 are deleted
Combined heterozygotes	[ ]+[ ]	[38A>C]+[77_79del]	In one allele of the gene, nucleotide 38 is changed from A to C and in the second allele of the gene, there is a deletion of three nucleotides form 77 to 79

Mutations at RNA level are generally described using the same rules as for DNA, except that the description of a mutation is preceded with "**r.**" that denotes for an RNA reference sequence (e.g. r.36u>a). Sequence changes are described in lower case letters using a (adenine), c (cytosine), g (guanine) and u (uracil) to describe bases, e.g. r.36u>a describes substitution of uracil at positons 36 with adenine. If a mutation results in two or more transcript production it should be described in brackets, e.g. [r.=, 73_88del] denotes that a nucleotide change in DNA results in production of one transcript of the normal sequence (r.=) and the second transcript with a deletion of nucleotides from 73 to 88 [3].

Mutations at protein level are generally described using the same rules as for DNA, except that the description of a mutation is preceded with "**p.**" that denotes for a protein reference sequence. Sequence changes are described in capitals and one letter amino acid code is preferred. Numbering of amino acids begins with the first methionine (+1 amino acid). See Table 2 for general recommendations for description of mutations at DNA levels.

Table 2. General recommendations for description of mutations at protein level. Based on ref. [3].

Type of change	Sign	Example	Description
Substitution		p.K20C	Missense change; lysine 20 (K) is changed to cysteine (C)
	X (stop codone)	p.K20X	Nonsense change; lysine 20 (K) is changed to a stop codon (X)
Deletion	del	p.K20del	Deletion of one amino acid (lysine, K) at position 20
		p.K20_M22del	Deletion of three amino acids from lysine 20 to methionine 22
Duplication	dup	p.G31_C33dup	Three amino acids from glycine 31 to cysteine 33 are duplicated
Insertion	ins	p.K20_M21insQ	Insertion of glutamine (Q) between amino acids lysine 20 (K) and methionine 21 (M)
		p.K20_M21insQCS	Insertion of three amino acids glutamine (Q), cysteine (C) and serine (S) between amino acids lysine 20 (K) and methionine 21 (M)
Initiating methionine (M1)	p.?	p.?	The effect of mutation on the protein level is unknown.
	p.0	p.0	The mutation results in no protein production
Frameshift mutation	fs (or fsX)	p.R67fsX78	fs is placed after the first affected amino acid and the new reading frame ends with the amino acid 78.
Variability of short sequence repeats	( )x-y	33(Q)6-9	The amino acid glutamine 33 is repeated 6 to 9 times in the population

1.3. Samples for genetic testing. Collecting, transport and storage

A wide range of biological samples may be used as a source of genetic material for molecular diagnostics. The most common type of sample obtained from an adult individual is blood sample which is a very good source of DNA or RNA for molecular diagnostics. Alternative to blood samples are Mouthwashes or buccal scrapes that may be collected very noninvasively and these samples are useful in population screening studies. To obtain fetal DNA, chorionic villous biopsy or amniotic fluid samples can be used. One or two cells removed from eight-cell stage embryos are used in case of pre-implantation genetic testing during *in vitro* fertilisation procedures (IVF). Dried blood spots (Guthrie card samples), i.e. blood samples blotted and dried on filter paper, are used in neonatal screening for genetic disorders. Tissues from biopsies or cultured cells are also used as a source of target DNA or RNA. Archived pathological specimens (e.g. Formalin Fixed Paraffin Embedded Tissue, FFPE)

are used in molecular testing of tumours or to obtain DNA of dead people. Hair or semen are used for molecular studies in criminal investigations [4, 5].

Before any genetic testing may be performed, an **informed consent** must be obtained form all participants and they need to be informed about any current, future and unforeseen usage of their samples.

Stability of collected biological samples may be affected by many different factors, e.g. anticoagulants in collection tubes, stabilizing agents, temperature, timing before initial processing takes place, sterility, endogenous degrading properties (enzymes, cell death) etc. [5]. For example, citrate-stabilized blood may afford better quality of RNA and DNA than other anticoagulants would, also, EDTA is good for DNA-based assays, but it will influence Mg^{2+} concentration, and it may interfere for example with cytogenetic analyses [5].

Many biochemical biomarkers may be affected by enzymatic degradation. Proteins are sensitive to degradation by proteases. To prevent degradation of proteins, specific commercially available protease inhibitors (e.g. aprotinin, pepstatin, antipain, leupeptin, or benzamidine) should be added immediately after collection of samples. Additionally, handling with proteins must be lead on ice. RNA is sensitive to degradation by RNases, thus RNase inhibitors should be added immediately after collecting before the sample will be processed. RNA stabilization reagents are available commercially (e.g. RNA later) and are very useful, if RNA isolation needs to be postpone for days, weeks or months after specimen collection. The reagent immediately stabilizes RNA in samples and preserves the gene expression profile. Additionally, all steps of handling with RNA require RNAse-free containers, equipment, enzymes etc. to prevent degradation and are important for maximizing the yield and quality of RNA preparation [5].

In case of some tests available only in certain reference centres, collected samples must be transported over long distances. To obtain a high quality DNA or RNA, collected biological samples should be stored in proper conditions. Depending on the type of sample to be shipped, samples may be shipped on wet ice or even at room temperature if shipped overnight [5].

Temperature affects sample stability during the time between sample collection and sample processing and during short and long-term storage. For example, isolated DNA may be stored at +4°C for several weeks, at -20°C for several months and isolated RNA must be stored at -80°C. Live cells are stable at room temperature for up to 48 hours but must be either cultured or cryopreserved in liquid nitrogen. Finally, long-term storage (for many years) may affect the integrity of nucleic acids. Both DNA and RNA must be stored at -80°C. Multiple aliquots of a sample are necessary, to avoid repeated freezing and thawing in order to prevent loss of the entire sample due to cross-contamination. Importantly, frost-free freezers must be avoided because they cause small volumes of aliquots to dry, even in capped tubes [5].

1.4. Methods in molecular diagnostics of human genetic disorders

Some molecular diagnostic methods are used to test for presence of a specific, known DNA sequence change within a gene. These methods include for example PCR-RFLP, ASO, ASA, OLA and are especially useful in testing for genetic disorders showing limited allelic heterogeneity, in molecular diagnostics within a family or in research population studies. But some other genetic disorders are characterized by allelic heterogeneity, thus mutation screening methods, that test for any mutation, anywhere within the specific gene, are frequently used in the first place in molecular diagnostics. Most commonly used screening methods include SSCP and heteroduplex analysis. Screening diagnostics may be followed by sequencing of the gene to seek for the exact nucleotide change (mutation) [4].

1.4.1. Restriction Fragment Length Polymorphism Analysis of PCR-Amplified Fragments (PCR-RFLP)

PCR-RFLP is a method that couples PCR amplification with restriction analysis of produced DNA fragment. This technique, also known as cleaved amplified polymorphic sequence (**CAPS**), is very useful in genotyping. Single nucleotide polymorphisms (SNP) often create or abolish specific restriction sites, thus a specific nucleotide variation in this position may be detected using restriction endonucleases that recognize such sites. First, a specific fragment of a gene containing the variation is amplified by PCR. Next, the fragment is digested with an appropriate restriction enzyme and digestion products are resolved by agarose or acrylamide gel electrophoresis [6]. Depending on the presence or absence of investigated sequence variation, the PCR products digested by restriction enzyme will give readily distinguishable patterns after electrophoresis (Fig. 1).

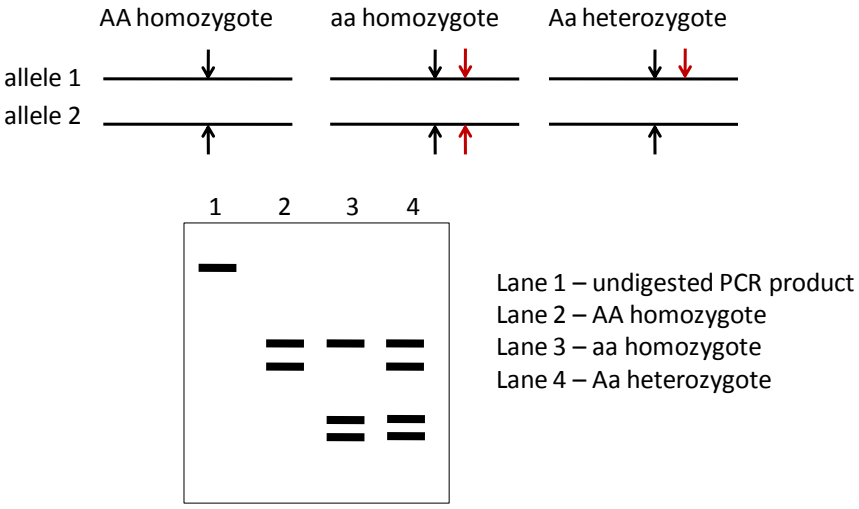

Fig. 1. An example of genotyping based on PCR-RFLP assay.

A modification of previously described CAPS method is Derived Cleaved Amplified Polymorphic Sequences (dCAPS) method where PCR primer possesses a mismatched nucleotides in order to create a restriction site if a specific nucleotide change occurs in the target sequence.

Genotyping based on PCR-RFLP is quite inexpensive and easy to design (there are programs for the design of PCR-RFLP), which is an advantage of this method, but the exact genotyping cannot be achieved if there is more than one nucleotide variation in a restriction enzyme recognition site. The procedure is relatively time consuming, because the full analysis consists of several steps [6].

1.4.2. Allele Specific Oligonucleotide (ASO) hybridization

Allele specific oligonucleotide (ASO) analysis is a hybridization method for detection of point mutations or few base pair changes. Oligonucleotide probes used in ASO hybridization comprise 15-20 nucleotides and are complementary to sequences spanning the variant nucleotide site. Hybridization is run in very strict conditions so that the probe hybridizes with target DNA sequence only if there is a perfect base matching. If a single mismatch between probe and target DNA occurs (Fig. 2), the probe-target heteroduplex is very unstable at highly strict hybridization conditions and detection is possible only if there is a perfect match between probe and target. Oligonucleotide probes are labelled with radioactive, enzymatic or fluorescent tag to enable further detection of hybridization [4, 7].

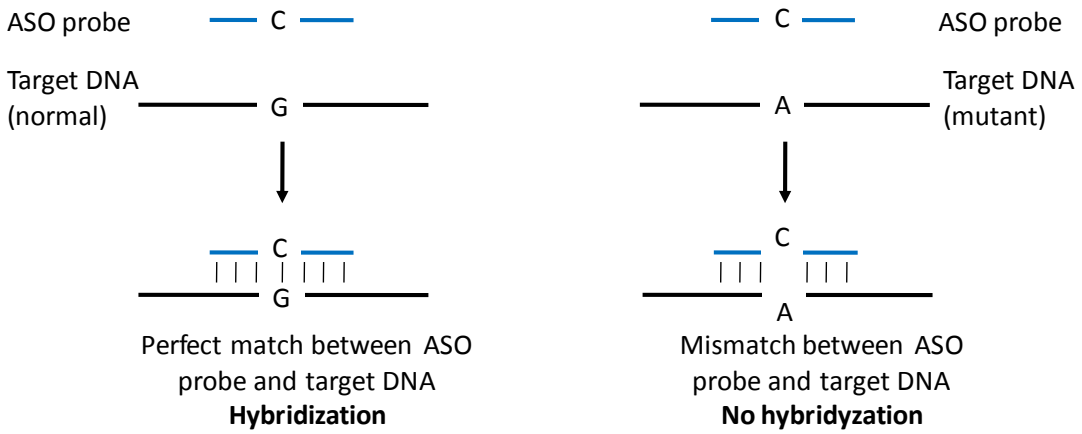

Fig. 2. Alelle specific oligonucleotide (ASO) hybridization scheme.

Allele-specific oligonucleotide (ASO) hybridization is rapid and efficient method for screening of known and most common mutations in a specific gene. For example in cystic fibrosis, ASO hybridization is used to detect the most common mutation F508del in the *CFTR* gene. Two ASO probes spanning the mutation site are designed, one complementary to the normal allele and the other to the mutant allele (i.e. sequence with deletion of AGA

nucleotides). The PCR-amplified genomic DNA of tested individuals is spotted in duplicate onto a dot-blot hybridization membrane, next, either normal or mutant ASO probe is added and hybridization is detected, depending on the labelling tag of the probe (Fig. 3). Similarly, ASO hybridization is commonly used to detect the point mutation at codon 6 (GAG to GTG) in the *HBB* gene coding for β-globin in sickle cell anaemia.

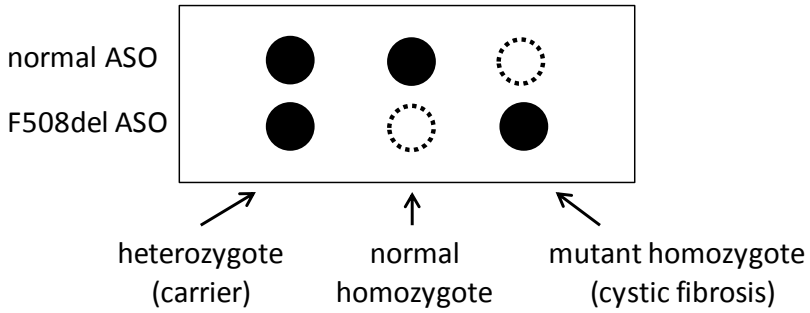

Fig. 3. Schematic dot-blot hybridization of allele specific oligonucleotide (ASO) in detection of the most common mutation F508del in cystic fibrosis. Black dot denotes hybridization of target DNA sequence with the probe and clear dot denotes no hybridization of target DNA sequence with the probe.

1.4.3. Allele-specific amplification (ASA)

Allele-specific amplification (ASA), also called allele-specific PCR (AS-PCR) is a PCR method used to detect known point mutations or known polymorphisms at the same locus. The method bases on the fact, that only a primer with exactly the same sequence as the target DNA will be amplified and primers that have a mismatch at the 3' terminal nucleotide will not be elongated during PCR reaction. Allele-specific primers are designed to match, at their 3' ends, the two variants (A/B) at the polymorphic site. The genotyping is run in two complementary PCR reactions, one containing one allele-specific primer (e.g. wild-type specific) and the common reverse primer and the other containing the other allele-specific primer (e.g. mutant specific) and the common reverse primer (Fig. 4). DNA polymerase used in this assay must lack the 3'→5' proofreading activity, so as to not eliminate the 3' mismatch. Described detection method based on AS-PCR is also known as amplification refractory mutation system (ARMS) [4, 8].

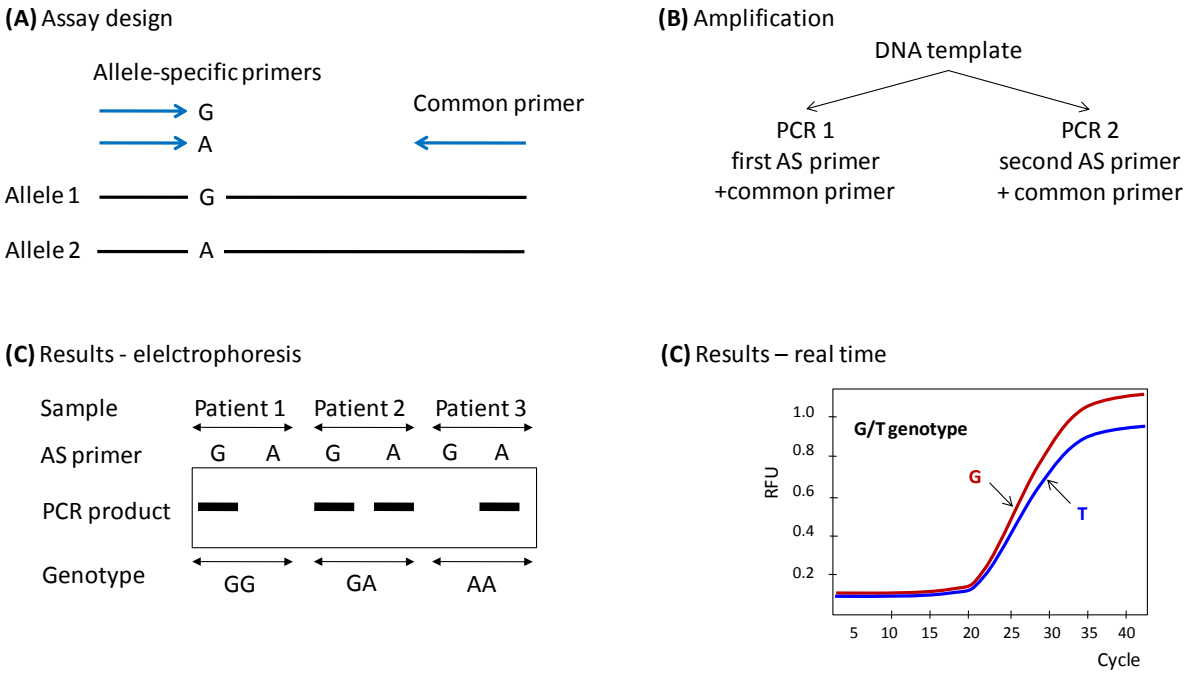

Fig. 4. Scheme of allele-specific amplification method. Assay design (A), amplification work flow (B) and possible results obtained by electrophoresis (C) or real-time amplification (D) are presented.

Different variants of ASA are currently in use. For example in tetra-primer ARMS-PCR method four primers are used in a single PCR reaction, i.e. one pair of inner allele-specific (AS) primers and one pair of outer standard primers designed in such a way that the amplicons of two alleles differ in sizes and can be resolved by electrophoresis. In allele specific primer, near its 3'-end, an additional mismatch may be incorporated in order to improve specificity and reliability of ASA. The single nucleotide polymorphism (SNP) genotyping assay based on allele-specific amplification may be combined with real-time monitoring. Two separate amplifications for the sample, each with one allele-specific primer (wild-type or mutant specific) are run with SYBR Green dye and fluorescence is measured by the instrument in every amplification cycle to detect the increase of the PCR product amount. If an allele is not present, no fluorescence will be detected [4, 8].

1.4.4. Oligonucleotide Ligation Assay (OLA)

An oligonucleotide ligation assay (OLA) is a rapid and sensitive method for detection of known single-nucleotide polymorphism (SNP) alleles. The gene of interest is first PCR amplified (in some applications genomic DNA may be used as a template in OLA). The DNA template for OLA assay is denatured to form a single-stranded target DNA. Three different oligonucleotide probes are used in OLA assay, two Capture Oligo probes, one for each genotype, and one Reporter Oligo probe that is modified with phosphate at 5'end (Fig. 5). Capture Oligo probes are colour coded, i.e. are labelled with two different fluorescent dyes

(one for each genotype) and one of them may be additionally size-coded by different length of the probe to differ both genotypes during electrophoresis. Oligonucleotide probes anneal to their complementary sequences in target DNA and DNA ligase joins two adjacent probes at the polymorphic site. Ligation products, labelled with different fluorescent dyes depending on the genotype, are separated by capillary electrophoresis coupled with fluorescence detection. Different detection formats may also be used (e.g. oligos labelled with biotin), nevertheless, main principles are the same [4, 9].

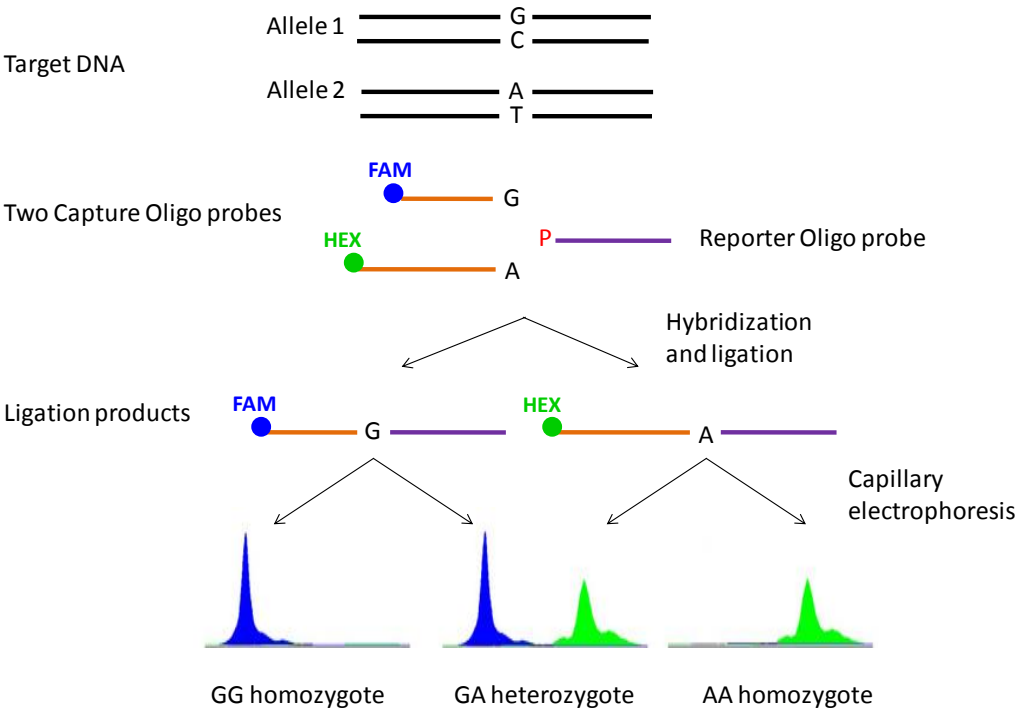

Fig. 5. Oligonucleotide ligation assay (OLA) with fluorescently labelled probes. Scheme represents an example of results obtained by capillary electrophoresis of ligation products followed by laser excitation and detection of fluorescence emission by CCD camera. Adapted from ref. [9].

1.4.5. Multiplex Ligation-dependent Probe Amplification (MLPA)

Multiplex ligation-dependent probe amplification (MLPA) is a quantitative method for detection of abnormal copy number, resulting from deletions or duplications of certain DNA fragments. An advantage of MLPA is that up to 45 different DNA fragments may be analyzed in one multiplex reaction. Additionally, in MLPA very small (50-70 nt) sequences are targeted, thus the method enables identification of single gene aberrations which are too small to be detected for example by FISH. The standard MLPA procedure consists of following steps: (a) denaturation of genomic DNA, (b) hybridisation of MLPA probes, (c) ligation of between two oligonucleotides, (d) PCR amplification of probes, (e) separation of PCR products by capillary-based or sequence gel-based electrophoresis and product

quantification, (f) analysis of collected data. Each MLPA probe consists of two oligonucleotides that hybridize to adjacent sequence of target DNA. Both oligonucleotides can be ligated to each other only if they hybridized to a target sequence, and the ligation product is a template for the following PCR amplification. Both oligonucleotides of MLPA probe consists of two regions, i.e. a region complementary to an universal primer and a region complementary to the target DNA, but the second oligonucleotide is longer and additionally, between these two regions, has a stuffer sequence with a unique length (Fig. 6A). Such MLPA probe design allows us to use only one pair of universal primers in multiplex PCR for all MLPA probes used in hybridization. Differences in length of the stuffer sequence between different MLPA probes enables identification of various amplicons produced in multiplex PCR using capillary electrophoresis separation (Fig. 6B). The forward primer used in PCR amplification is fluorescently labelled, thus each amplicon is detected by the means of fluorescence measure. The peak area or peak height of each amplicon reflects the relative copy number of the target sequence, thus detection of deletions (reduced signal) or duplications (increased signal) of regions of interest is possible (Fig. 6C) [10, 11].

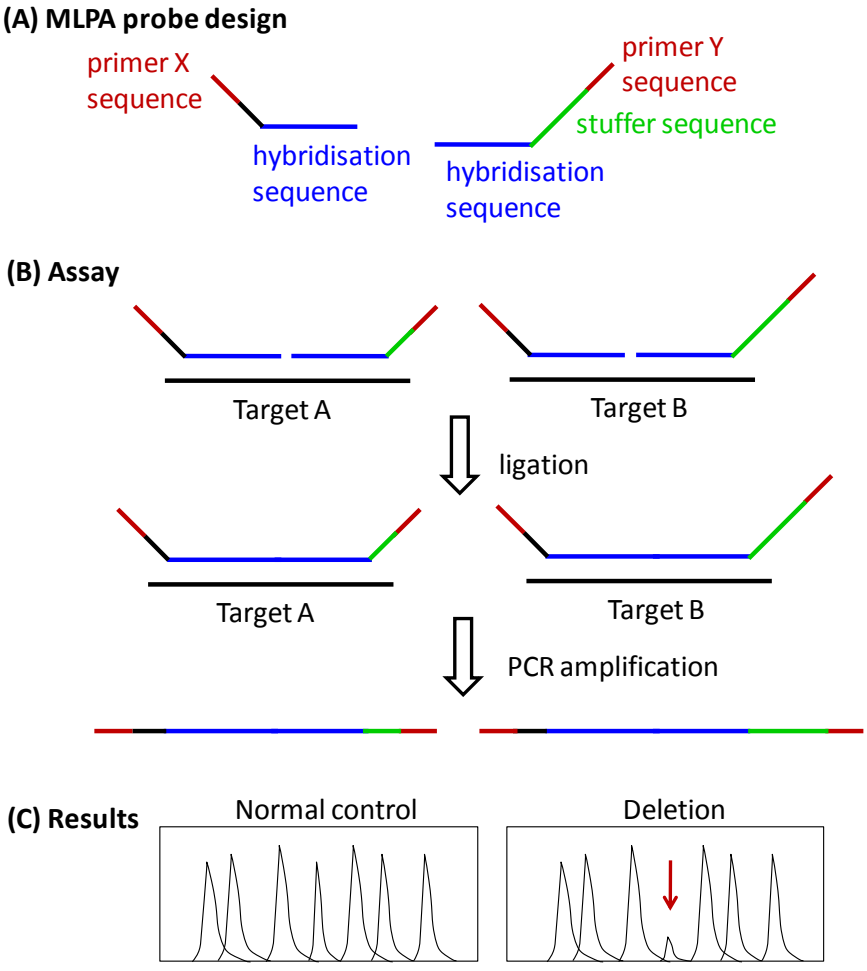

Fig. 6. Multiplex ligation-dependent probe amplification (MLPA). Adapted from ref. [10].

1.4.6. Single-strand conformation polymorphism (SSCP)

Single-strand conformation polymorphism (SSCP) is a method based on electrophoresis used for rapid detection and screening of point mutations in DNA fragments. The SSCP procedure involves amplification by PCR of a specific DNA fragment, that, next, is denatured by high temperature in presence of denaturing agents such as formamide, urea or sodium hydroxide. Denatured fragments are separated by polyacrylamide gel electrophoresis in nondenaturing conditions. The SSCP method uses the property of single-strand DNA (ssDNA) to form in a solution, under certain conditions, secondary structures called **conformers**. Formation of a specific structure depends on the nucleotide sequence of the DNA fragment and this process may be altered by nucleotide substitutions. Small differences in DNA sequence (often a single base pair substitutions) result in different secondary structures, thus affect the mobility of different conformers through a gel during electrophoresis (Fig. 7).

Standard detection methods use silver staining or radioisotopes. High throughput mutation detection using SSCP technique may be obtained by capillary electrophoresis and detection of fluorescently labelled DNA fragments. Apart from the detection method, fragments with the same nucleotide sequence form the same conformers with the same electrophoretic mobility, thus a specific band (or peak) pattern after electrophoresis is observed. Conformers formed by DNA fragments carrying a mutation are different and exhibit different electrophoretic mobility and the band (or peak) pattern differs comparing to DNA with the wild type sequence (Fig. 7).

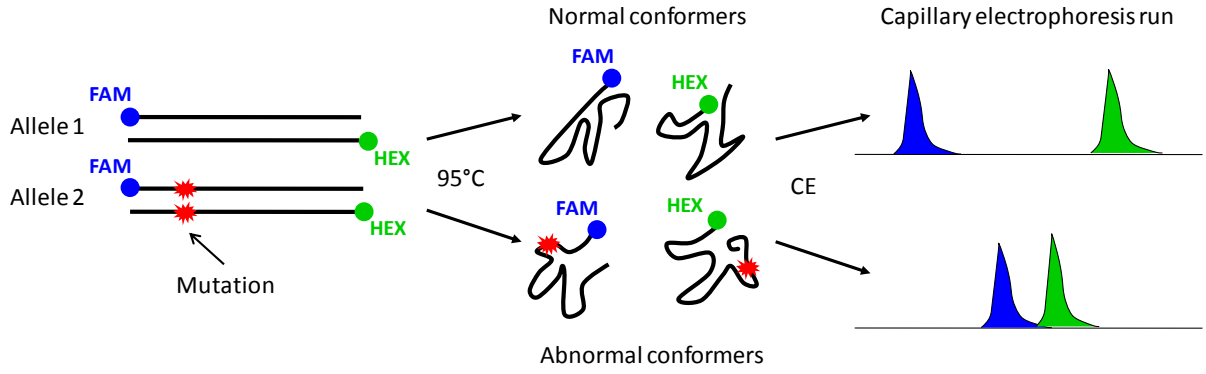

Fig. 7. Single-strand conformation polymorphism (SSCP) assay performed with PCR products labelled with different fluorescent dyes. Depending on the sequence, conformers differ with the electrophoretic mobility during separation by capillary electrophoresis (CE). Adapted from ref. [12].

Optimal size of PCR-amplified DNA fragments should be in the range of 150-300 bps. In this case, the method detects about 80-90% of potential sequence variations. For DNA fragments in the rage of 300-450 bps detection rate decreases 80% or lower. Additionally, the quality and efficiency of conformers formation depends on many factors such as temperature, DNA concentration, ionic strength, gel crosslinking and glycerol concentration

in the gel. Nevertheless, the SSCP is an inexpensive and sensitive technique, suitable as a scanning method for detection of unknown point mutations in a selected gene fragments.

1.4.7. Heteroduplex analysis (HET)

Heteroduplex analysis (HET) is a screening method to detect sequence variations in diploid DNA. DNA from target genes is amplified by PCR followed by a denaturation step. During slow renaturation, DNA strand hybridize randomly, i.e. DNA strands with complementary sequences (either both with the wild type sequence or both with the mutant sequence) hybridize to form homoduplexes, and non-complementary DNA strands (i.e. one with the wild type and the other with the mutant sequence) hybridize to form heteroduplexes (Fig. 8). Due to a mismatch in the site of a mutation, heteroduplexes differ from homoduplexes with conformation, electrophoretic mobility and melting temperature. After PCR, heteroduplexes may be analyzed by separation techniques such as conventional gel electrophoresis, denaturing high performance liquid chromatography (dHPLC), temperature-gradient capillary electrophoresis (TGCE) or recently heteroduplexes may be detected directly after PCR by high-resolution melting analysis. Some of the heteroduplex detection methods are described underneath.

1.4.8. Denaturing High Performance Liquid Chromatography (DHPLC)

Denaturing high performance liquid chromatography (DHPLC) is a method for identification of mutations based on heteroduplex formation between wild-type and mutated DNA strands. In DHPLC, heteroduplex molecules are separated from homoduplex molecules by ion-pair, reverse-phase liquid chromatography on a special column matrix in gradient of denaturing agent and temperature. Heteroduplexes and homoduplexes differ in stability under denaturing conditions, thus due to differences in retention of DNA on column matrix, heteroduplexes are washed out before homoduplexes and the elution profile is characteristic for the mutation change (Fig. 8). DHPLC method is characterized by about 100% sensitivity, thus it is very useful for the screening of a large number of samples for mutations, especially in molecular diagnostics of hereditary cancers characterized by heterozygous mutations in suppressor genes where heteroduplex analyses may be applied [13].

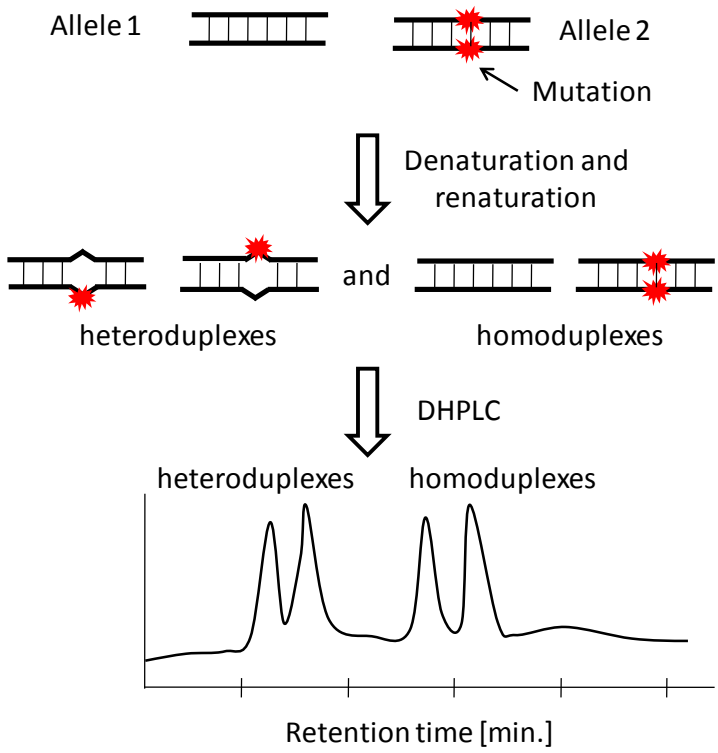

Fig. 8. Denaturing high performance liquid chromatography (DHPLC).

1.4.9. High resolution melting (HRM)

High resolution melting (HRM) is a post-PCR method, that enables analyses of genetic variations (SNPs, mutations, methylations) in PCR amplicons. In this method, the DNA region of interest is first amplified by PCR and in the next step, while the temperature rises from 50 to 95°C, melting of PCR products is conducted. Depending on the sequence variation (i.e. mutation or polymorphism within the target sequence), the melting curve of variant amplicons is slightly different. Either labeled primers or a saturating DNA dye may be used in PCR amplification to detect a change in shape of the fluorescent melting curve. With a high quality HRM assay, it is possible to distinguish between heterozygote, recessive homozygote and dominant homozygote at the locus of interest (Fig. 9).

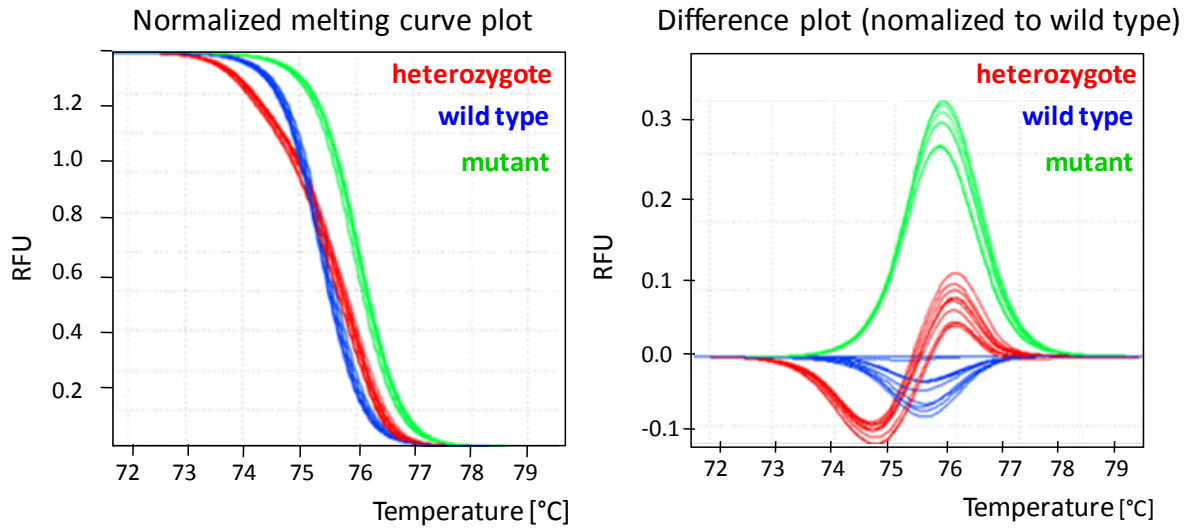

Fig. 9. High resolution melting (HRM) discriminating between wild-type homozygous, mutant homozygous and heterozygous genotypes. Adapted from ref. [14].

1.4.10. DNA sequencing

Sequencing is a method for determining the nucleotide sequence of DNA molecule, thus it is a very precise method for detection of exact mutations responsible for specific genetic disorders. Basic sequencing methods, both developed in 1977, include:

- Maxam-Gilbert sequencing, a method based on chemical modification of DNA followed by cleavage at specific bases
- Sanger dideoxy sequencing, a method based on selective incorporation of chain-terminating dideoxynucleotides by DNA polymerase during in vitro DNA replication.

The Sanger dideoxy sequencing method become more dominant because it was more convenient comparing to Maxam-Gilbert method and the automation of the sequencing process has been developed (in automated DNA sequencer instruments). In general, Sanger sequencing method involves following steps: (a) isolation of DNA, (b) PCR amplification of the DNA fragment of interest, (c) cycle amplification using mix of standard nucleotides and dideoxynucleotides, (d) gel electrophoresis or capillary electrophoresis of amplified products. When a dideoxynucleotide incorporates at the 3' end of synthesized DNA chain, the elongation terminates at A, T, C or G depending on the specific dideoxynucleotide that was incorporated. Automated sequencing technologies involve the usage of fluorescent dye-labelled dideoxynucleotides in cycle sequencing reaction. Each of four dideoxynucleotides (ddNTPs) is labelled with a different fluorescent dye, thus the reaction generates fragments of increasing size, ending with one of four dideoxynucleotides and the exact base is determined by the fluorescence colour detected by the sequencing instrument.

In 1996, a new sequencing technology, called pyrosequencing, has been developed by Mostafa Ronaghi and Pål Nyrén at the Royal Institute of Technology in Stockholm [15]. **Pyrosequencing** is a real-time sequencing method and, as distinguished from Sanger dideoxy method, pyrosequencing is based on detection of pyrophosphate release during nucleotide incorporation and not chain termination resulting from dideoxynucleotides incorporation. Four enzymes are involved in the pyrosequencing reaction, i.e. DNA polymerase, ATP sulphurylase, luciferase and apyrase. The method detects pyrophosphate (PPi) release accompanying DNA synthesis process. Following enzymatic reactions utilized the released pyrophosphate to generate a detectable light emission. The templates for pyrosequencing reaction are ssDNA fragments (which usually is one strand of PCR product obtained by amplification with biotinylated primer). Such strand binds to streptavidin-coated beads in order to remove the other strand by rinsing the bead. The sequencing primer is added and the template is introduced into pyrosequencer where the mixture of four enzymes is added. Next, dNTPs are added to the reaction sequentially. In a single cycle, one of four deoxynucleoside triphosphates (dNTPs) is added with exception that instead of dATP, which is a substrate for the luciferase, dATPαS, a nucleotide not recognized by luciferase, is added to the reaction, to prevent nonspecific luminescence. After a specific dNTP is added, DNA polymerase incorporates the current dNTP only if it is complementary to the current position on the DNA template. This incorporation is associated with pyrophosphate (PPi) release. The next enzyme, ATP sulphurylase, quantitatively utilize PPi to produce ATP using adenosine 5´ phosphosulfate (APS). The formed ATP is next utilized by luciferase to convert luciferin to oxyluciferin with accompanying release of light quantum. The release of PPi is stechiometric to the number of nucleotides incorporated by polymerase, thus the more the same nucleotides are incorporated, the more PPi is released and the more intensive is the light signal. Regeneration of the reaction mixture after one cycle is lead due to activity of apyrase, an enzyme that degrades ATP and unbound nucleotides after before the reaction begins with the next nucleotide in the following cycle.

The automated Sanger sequencing method is considered as a first-generation technology, and recently it has been replaced by **high-throughput sequencing technologies** that are referred to as **next-generation sequencing** (NGS). Next generation sequencing technology has revolutionized genomic and genetic research. In NGS thousands or millions of sequences are read in parallel sequencing process, thus, this strategy is suitable especially for large-scale, automated genome analyses.

Different high-throughput sequencing technologies are available on the market: Massively Parallel Signature Sequencing (MPSS), Polony sequencing, 454 pyrosequencing (Roche), Illumina (Solexa) sequencing, SOLiD sequencing, Ion semiconductor sequencing, DNA nanoball sequencing, Heliscope single molecule sequencing, Single molecule real time (SMRT) sequencing. Three of them are described underneath. Although all these platforms differ in sequencing biochemistry, they all share similar work flows comprising of steps such as template preparation (DNA fragment library), immobilization of library fragments,

generation of clonally clustered amplicons and sequencing, but, depending on the platform used, all these steps may be achieved by different approaches [16-19].

454 pyrosequencing technology. Template preparation for 454 pyrosequencing involves randomly breaking of genomic DNA into smaller size fragments (DNA library), that next are ligated with short adaptors providing priming sites for further amplification and sequencing. Additionally, B-adaptor contains biotin at its 5'-end that enables the immobilization of DNA fragments on the surface of magnetic, 28-μm capture beads coated with streptavidin. One DNA fragment is captured on one magnetic bead. In the next step, DNA fragments are amplified by emulsion PCR (emPCR), a PCR in a water-oil mixture, that form a microreactor for each bead, thus the amplification of bead-bound DNA fragment takes place independently. After emPCR, hundreds of thousand clonally amplified copies of the DNA fragment are immobilized on the bead surface. The emulsion is broken and following enrichment, DNA beads are layerd onto a titanium PicoTiterPlate. Only one DNA coated bead enters one well on the plate. After enzyme beads and packing beads are added sequencing is performed by the pyrosequencing method (described previously). Luminescence intensity is detected in real-time by CCD camera [16-19]. This sequencing technology is sold by Roche.

Illumina (Solexa) technology. The technology is based on sequencing-by-synthesis method. The DNA fragment library may be obtained by any method and resulting library of DNA fragments up to several hundreds of bps is ligated with adaptors. Clonal amplification is made by bridge PCR, where forward and reverse primers are fixed to a solid surface inside the flow cell channels. DNA fragments are amplified after adding of mixture of PCR reagents. After amplification about 1000 copies of ssDNA fragments are created forming a surface bound colony (a cluster). Next, sequencing primer is annealed and sequencing reagents, containing four reversible terminator nucleotides, are added. Each nucleotide is labelled with a different fluorescent dye, thus after incorporation in the current position into DNA strand, the fluorescent signal is detected by CCD camera. In one cycle, only one terminator nucleotide is incorporated and before the next cycle begins, the 3' terminator group as well as the fluorescent dye of the currently incorporated nucleotide are removed and the synthesis cycle is repeated [16-19]. This sequencing technology is sold by Illumina.

SOLiD technology. The technology is based on sequencing-by-ligation method. Depending on the purpose, two different types of DNA fragment library may be produced, i.e. fragment library or mate-paired library. DNA fragments are ligated to adapters, next they are bound to beads and amplified by emulsion PCR. After amplification, DNA templates are modified at the 3' end, covalently bound do glass slide and the sequencing process by subsequent ligation steps begins. First, the sequencing primer is hybridized to adaptor sequence and next oligonucleotide octamers labelled with fluorescent dyes and the ligase are added. First two nucleotides of the octamer determine the colour of the fluorescent label. Three next positions are degenerate nucleotides (thus, 64 different versions for each

particular dinucleotide are available) and three last positions are universal bases, that are the same for all detector oligonucleotides. In the first ligation cycle with oligonucleotide octamers, fluorescence signal determines bases 1 and 2 in the sequence. Each ligation reaction provides information about colour of particular dinucleotide. Ligated octamers are cleaved after their fifth base that removes the fluorescent label. The next ligation cycle begins, in which fluorescence signal of the ligated octamer determines the bases 6 and 7. In the third cycle bases 11 and 12 are determined, etc. After a series of such ligation processes, the product is removed thus the template is reset and another sequencing primer is hybridized to the template DNA. Another round of octamer oligonucleotides ligation is initiated and the detected fluorescence signal determines bases -1 and 1 in the first cycle, bases 5 and 6 in the second cycle, bases 10 and 11 in the third cycle, etc. Once again, after a series of ligations, the product is removed, the template is reset and in the third round of ligations, bases 4 and 5, bases 9 and 10, bases 14 and 15, etc. are determined. Five rounds or ligation cycles are needed to determine the sequence. Because four different dinucleotides may correspond to the same colour, the analysis of the -1 nucleotide (which is a known oligonucleotide of the adaptor sequence) enables to determine the whole sequence [16-19]. This sequencing technology is sold by Life Technologies.

1.5. Biomarkers

According to National Institutes of Health (NIH), a **biomarker** is “a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention”. In a number of genetic disorders, along with molecular diagnostics, different biomarkers are used for screening, risk assessment, diagnosis or monitoring of treatment efficacy. Some biomarkers can determine staging or grading of a disorder or may be useful in selection of initial therapy. Different types of molecules may be used as biomarkers, i.e. DNA and RNA biomarkers, protein biomarkers or metabolites [20]. Examples of biomarkers of selected genetic disorders are listed in Table 3.

Table 3. Examples of biomarkers of selected human genetic disorders [20].

Disease	Biomarker	Description
Down syndrome	Biomarkers include β-human chorionic gonadotrophin (hCG), alpha-fetoprotein (AFP), unconjugated oestriol (uE3), serum pregnancy associated plasma protein-A (PAPP-A), dimeric inhibin A	Elevated levels of these biomarkers enable the prenatal diagnostics for Down syndrome
Muscular dystrophy	Creatine kinase (CK) blood levels	Diagnostic biomarker of several neuromuscular diseases, including DMD
	nNOS (neuronal form of nitric oxide synthase)	May be useful in suspecting a dystrophinopathy because sarcolemmal nNOS is lost when dystrophin levels are very low or absent [21]
	Muscle-miRNAs in serum (miR-1, miR-133, and miR-206)	microRNAs, specifically expressed in muscle cells, are released into the blood of DMD patients as a consequence of muscle degeneration and their amount correlate with the severity of the DMS disease; Cacchiarelli et al. proposed the use of muscle-miRNAs as a biomarker in diagnostics and possibly for monitoring the outcomes of therapeutic interventions [22]
Phenylketonuria	Phenylalanie and thyrosine	Quantitative measurement of phenylalanine and tyrosine in neonatal blood samples by tandem mass spectrometry
Niemann-Pick disease type A and type B	Cytokine MIP-1α in serum (tested in mouse)	A biomarker elevated in serum of a mouse model of Nieman-Pick disease and reduced to normal levels after enzyme replacement therapy [23]
Mucopolysaccharidoses	Heparin cofactor II-thrombin (HCII-T) complex in serum and dried blood spots	HCII-T is elevated up to 25-fold in MPS diseases that store dermatan sulphate and about 4-fold in MPS III that stores heparan sulphate comparing to unaffected age-matched controls, thus, HCII-T may serve as an effective biomarker for MPS I, II, VI and VII diseases [24]

Gaucher disease	Chitotriosidase,	Most useful markers to follow enzyme replacement therapy; chitotriosidase is an enzyme which is dramatically elevated in symptomatic Gauchers patients
	CC chemokine ligand 18 (CCL18)	Plasma levels of CCL18 are elevated in plasma or in urine of symptomatic patients with Gaucher disease and decrease during therapy [25]
Fucosidosis	Oligosaccharides	Elevated levels of oligosaccharides in urine may be detected by mass spectroscopy
Huntington disease	The most promising candidates: 24-hydroxycholesterol in plasma, BDNF (brain-derived neurotrophic factor) in serum, TGFβ (transforming growth factor β), interleukins (4, 5, 6, 8, 10, 12, and 23) in plasma or cerebrospinal fluid, clusterin in plasma or cerebrospinal fluid	Different candidates are tested to search a good biomarker for evaluation of disease progression in individuals at the premanifesting stage of the disease as well as for use in therapeutic trials to monitor the effects of treatment [26]

1.6. References

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2. Model organisms for human genetic disorders

Scientists are using model organisms to study health and diseases, but what are "model organisms"? In science, a model is a simplified system that is accessible and easily manipulated. So a model organism is a species (animal, plant or microbe) that can be used to study certain biological processes. This organisms has been widely studied, are easy to maintain and breed in a laboratory settings. They are used to obtain information about other species because basic principles of fundamental properties how cells grow, divide, how inheritance works, how organism store and use energy are nearly the same in all living things. Moreover by studying model organisms scientists are learning more and developed new methods for maintaining health, diagnose and treat diseases in humans. Up to date model organisms from phages to mice have taught us invaluable lessens about fundamental biological processes and disease-causing mutations.

We can distinguish three major types of model organisms and their characteristics are described in Table 4.

Table 4. Major types of model organisms

Genetic models	Experimental models	Genomic models
• Species that are susceptible to genetic analysis • They can be breed in large numbers and have a short generation time, large scale crosses can be set up and followed over several generations • Many different mutants are generally available and highly detailed genetic maps can be created	• Species that are not so easy genetically susceptible • They have longer generation intervals and poor genetic maps • These species are widely used in developmental biology, embryos studies and manipulation	• Species chosen as model organisms because they occupy a significant position in the evolutionary tree or because of some quality of their genome

When scientists discover that a particular gene is associated with a disease in humans the next step is to find out what that gene does in a model organism, which often lead to better understanding the cause of a disease and develop potential diagnostic tests and treatment. There are many examples how model organisms has an impact on human health. Research with bacteria, viruses and yeast bring to light how all living things copying and

passing on their genetic material as well as fixing mistakes that get made during this process. Studies on yeast let us to understand the sequence of events of cell cycle. This knowledge is very important because since than many drugs interfering with cell cycle has been discover and use to treat cancer. Yeast have been used also to study gene expression, and deliver information about how genes are turn on or off. Another example of crucial discoveries where studies in *Drosophila* and *C.elegans* lead to learning that genetically controlled cell death plays a critical role in cancer and other diseases [1]. Laboratory rats have been used to test drugs and genetically engineered mice have been used as a models of many human diseases. Some such species and their research applications are described in Table 5 adapted from [2].

Table 5. Models used to study genetic principles and human diseases

Model organism	Common name	Research applications
Saccharomyces cerevisiae	Yeast	• Cell cycle studies • Cancer research
Drosophila melanogaster	Fruit fly	• Gene mapping • Recombination studies • Mutant screens to identify genes related to specific functions
Caenorhabditis elegans	Nematode	• Development of nervous system • Aging process • Developmental biology
Danio rerio	Zebra fish	• Mapping and identifying genes involved in organ development
Mus musculus	Mouse	• Genetic principles and human disease
Rattus	Rat	• Genetic principles and human disease

Drosophila, *C.elegans*, yeast, bacteria and viruses can provide useful information on the fundamental biological role of genes but they cannot deliver the information about

effects on gene modification on the development of organs or physiological diseases processes that are only found in mammals. Rodents are the most common type of mammal employed in experimental studies. Extensive research has been conducted using rats, mice, gerbils, guinea pigs and hamsters. Among these rodents the majority of the studies have been performed on mice. It is difficult to make an accurate estimation of the total number of mouse mutant lines available in the world today but its more than 3000 [3].

2.1. Animal models of human genetic diseases

Choice of available models to medical genetics is wide and with the advances in molecular genetics animal models of human diseases are becoming more numerous every day. Due to the remarkable level of homology between genomes of different organisms across evolutionary tree we can learn a lot about molecular mechanisms associated with single-gene diseases in humans by studying much simpler organisms such us mice. Similar to that of humans, mouse genome had been entire sequenced and many human genes have homologs in mice. Naturally occurring animal models of human genetic diseases are rare but that kind of mutation can be introduced into corresponding mouse gene using genetic engineering technics. We are able to create mice with a mutation or deletion of gene responsible for certain disorder [4]. It is also possible to insert human genes into the genome of mice (humanized mouse models) to study their physiological role. This mice permit functional *in vivo* studies of human cells and tissues [5]. Creating different kind of models allows us analyzed in details the development, physiology and biochemistry of a particular disease. A summary the contribution of mouse models in different areas of disease research is presented in Table 6 (adapted with modifications from http://www.nuffieldbioethics.org).

Table 6. Mouse models in different areas of disease research

Disease	Mouse model	Outcome and limitations
Diabetes	• NOD mouse type I diabetes [6] • db/db mouse type II diabetes [7]	• research on genetic pathways involves in diabetes and the hormonal and metabolic control of blood sugar level
Obesity	• mutants available that contribute to obesity under different conditions [8]	• research on the hormonal (leptin) and hypothalamic pathways of obesity
Neurological	• mutants available that affect neuronal growth, differentiation and plasticity [9]	• information about genes involved with the development of neuronal process, testing new therapeutic approaches to neurological diseases
Neurobehavioral	• mutants available of disturbance in complex behavior i.e. learning, memory, anxiety, aggression, feeding, circadian rhythm [10-12]	• there are no direct models of complex behavior but there are many strains with the sympthoms similar to psychiatric disease in human
Sensory	• mutants available with defects in hearing and vision[13, 14]	• there are many useful models of retinopathies and deafness
Cardiovascular	• several mutant models available [15-17]	• some progress in the study of atherosclerosis and hypertension
Cancer	• many mutants and strains of mice which show significant variation in both frequency and types of cancer[18-20]	• tumor formation in the mouse does not always correspond to that in humans but a lot of drug tests and research on the role of genes that are responsible for causing cancer in mammals
Musculoskeletal	• many myopathy models [21] and skeletal disease [22]	• information about genes involved with myopathies and better understanding of that disease and test potential therapies
Disorders related with age	• mutants available for Alzheimer's [23] and Parkinson's disease [24]	• better understanding of that and other neurodegenerative disorders and test new therapeutic options

Animal models have an inborn or induced pathology that is equal or homological to that one observed in humans, and this is the main reason why models of human genetic diseases are so helpful in that type of research. Most of the genetic diseases are very rare

and because of that it is very difficult to perform studies on patients. In the opposite to that animal model for the rare disease can be bred and used when desired. Moreover it give us easy access to samples of tissues and biological fluids of any kind and observations can be made at all stages of both the disease and the development of the animal. That kind of advantage is especially important for study late onset diseases because we are able to look at early pathophysiology before the symptoms occur thus lead us to better knowledge of early pathogenesis. Another reason why models are necessary is the ethical issue e.g. when we would like to test drugs or therapies it is impossible to do it directly on humans. When a model is available it is possible to compare groups of treated and untreated animals over the course of different protocols and treatments. Finally, animal models are important because of them it is possible to establish a link between a mutant gene and an abnormal phenotype sometimes by identification of an abnormal protein function.

Generally we can classified animal models into one of two categories:

I. **Homologous** – when the phenotype results form a genetic alteration in the orthologous genes in both species
II. **Analogous** – the phenotype is similar but results from genetic alteration in different genes

It has to be point out that that kind of classification does not mean that one category of models is better than the other, and we have to remember that mutation at the same genetic locus in different species can give different phenotypes and maybe it is better to use models with the similar pathologies even if they result from different genetic mutation. Everything up to what scientific question we would like to address.

2.2. Frequent human genetic diseases and their animal models

Up to date in many animal clinics we have got a large source of models of human genetic diseases. One of them is human obesity syndrome which is a heterogeneous autosomal recessive disorder. There have been identify a number of genes in which missense or nonsense mutation are sufficient to results in severe early onset obesity thus to disruption of normal appetite control mechanism (hyperphagia) [25]. There are two animals models which are object of intensive researches with similarities to that kind of syndrome in humans. The mutant alleles are *obese* (*ob, Lep^{ob}*)[26] and *adipose* (*ad, Lepr^{ad}*)[27]. This two

mutation have been characterized in the mouse, and this studies give a lots of information about protein *leptin* and its receptor [28]. This protein is a 16-kDa cytokine, which is synthesized in adipocytes. Serum level of it regulates the amount of energy stored in fat tissue as well as energy consumption. The mouse models of leptin deficiency are useful tools to investigate in detailed the role of this protein as well as possible use of it as a drug for obesity. In the Fig. 10 is an example of a mouse model with a mutation in *Lep* gene.

Fig. 10. Mouse homozygous for the mutation in *Lep* gene and their control littermates. The mutant mice have no functional receptor for the cytokine leptin or do not have it at all. Animals do not control their satiety and because of that grows very fat. Several alles have been described in this locus with similar phenotypes. Depending on genetic background that mice can also be diabetes. Adapted from [29].

Another interesting mouse model is homologous model of alkaptonuria [30](Fig.11), which is a rare metabolic disorder inherited in autosomal recessive manner. The obvious clinical feature of that disease is color of the urine, because its turn black when exposed to air. That kind of situation occurs due to the defect in a gene coding HGD (homogentisate 1,2-dioxygenase) enzyme which is localized in the 3 chromosome (3q13.33 – OMIM ID#607474). The symptoms of the diseases are identical between mice and human but there is one exception. Patients suffering from this diseases have ochronosis, thus consequence of the accumulation of crystals of homogentisate 1,2-dioxygenase in connective tissues which lead to arthritis in elderly specially in the spine and large joints. Ochronosis does not develop in mice, however the localization of the genetic defect in the mouse model allowed the identification of the chromosomal location in humans [31], as well as its very helpful future therapeutic developments.

Fig. 11. Mouse on the right suffers from alkaptonuria because its gene encoding enzyme HGD is non-functional. Adapted from [29].

Up to date we have many mouse models for metabolic disorders and they are invaluable useful tool to study molecular genetics, pathology and potential therapeutic options for that type of disease. One of the examples is ferrochelatase deficiency (Fig.12) which is a genetic disorder inherited in recessive manner and results in severe erythropoietic protoporphyria. Mutation which cause that phenotype is T to A transversion leading to methionine-lysine substitution at position 98 (M98K) of the protein ferrochelatase. This point mutation results in severe deficiency in FECH activity (about 2.7-6.3 % of the normal level). Lack of the enzyme activity results in hemolytic anemia, photosensitivity, cholestasis and severe hepatic dysfunction [32, 33].

Fig. 12. Mouse model with ferrochelatase deficiency causing anemia, photosensitivity, cholestasis and hepatic dysfunction, this mutation represents a model for human erythropoietic protoporphyria and the severity of the syndrome is influenced by genetic background. Adapted from [29]

Another interesting example is a mouse model of Marfan syndrome. All mice homozygotes for spontaneous or targeted mutations at the Fibrilin1 locus (*Fbn1*) are lethal. They express a lot of abnormalities include vascular defects, excess bone growth, connective tissue hyperplasia and lung emphysema leading to death from embryonic to around 4 months. Phenotypic expression of the same genetic defect is very strain dependent that suggest existence of influence of some genetic modifiers. Fortunately there are models (Fig. 13) with phenotypic similarities to Marfan syndrome but different etiologies [34].

Fig. 13. Mouse model with recessive mutation *strigosus* (*Npr3*^{stg}) with growth abnormality, extraordinary long body similar to Marfan syndrome. The phenotypic pathology get worse and animal become extremely thin with severe hunchback and elongation. Adapted from [29].

We still have difficulties to create animal models with chromosomal abnormalities. Mouse trisomies are easy to generate but none of them model any on the human disorders because the gene distribution along the chromosomes is different between this species. One of the most common trisomy in human population is Down syndrome, so it would be very useful to possess a model of this genetic alteration. Fortunately once again we can mimic symptoms of the disease and good and relatively accurate models of Down syndrome have been engineered. Transgenic mice with large insert of HSA21 have been creating [35, 36]. Human chromosome 21 shares a large region of genetic homology with mouse chromosome 16, but the trisomy of this chromosome is not a good model because the animals die in uterus. Moreover not all of the genes are similar because human chromosome 21 have also orthologs on mouse chromosomes 17 and 10. In Down syndrome there is a certain region (21q21.3-q22.2) which is called critical region. Within this region there is a locus for a gene which alteration is connected with learning defects and to create a good model for Down syndrome the attention has focused on that region.

The study of human diseases has advanced in large part due to the discovery or creation of immune deficient animal models. This animals are incapable of rejecting implanted tissues (xenografts) that means that human cell lines can be inserted and will continue to grow in these animals. Immune compromised mice have become a routine and valuable research tool in oncology. Cancer cell can be implanted and form tumors in the same way as it happened in humans. Nude or athymic mice and rats are missing the thymus gland, which is responsible for maturation of T lymphocytes. As a side effect this models are characterize by the lack of fur in opposite to Severe Combined Immunodeficiency Disease mice (SCID) (Fig.14A). SCID mice are unable to make either T or B cells, thus they are missing all of their immune response. These mice have a mutation on the 16 chromosome, and they are less likely to reject implanted cells than nude mice, since they are completely without immune system. Moreover we are able to generate models with additional alterations i.e. with green fluorescent protein (GFP) (Fig.14B), which allows us to have more closer look at the particular gene and the effects it can have on the physiology or what is the distribution over the body.

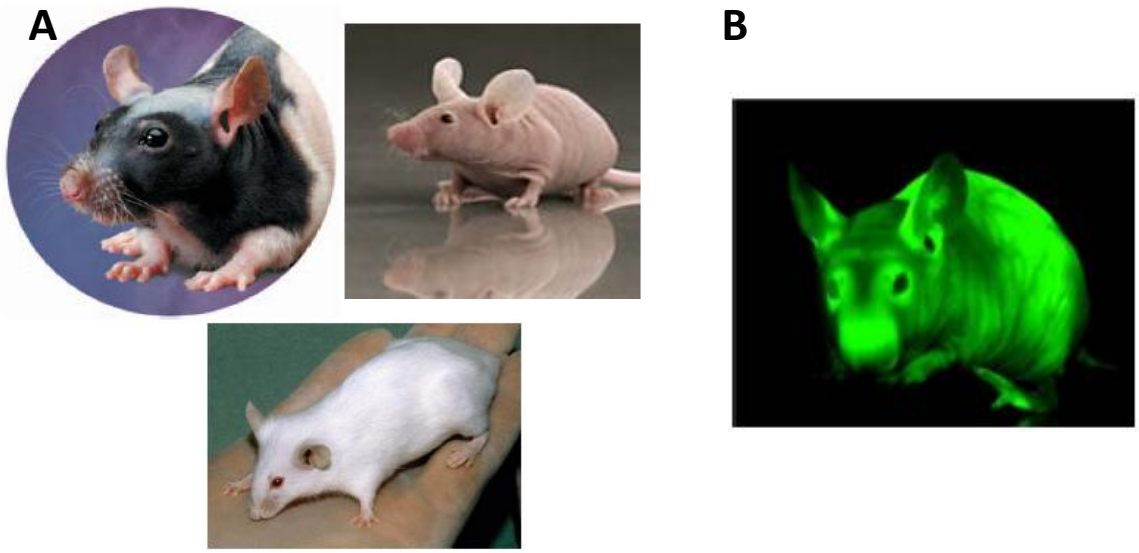

Fig. 14. Panel A presets Nude rat and mouse (on the top) and SCID mouse (on the bottom), panel B presents mice expressing GFP. Adapted from www.criver.com.

This chapter summarized potential role for studying human genetic and different disorders in some of model organisms. In all of them animal models have greatly improved

our understanding of the cause and progression of human genetic disorder and become a useful tool for testing potential therapeutic approaches. There are still the main question – how relevant to human diseases are in this animal models? We know that some of the genetic factors and pathways are shared between animal models and humans and the others are completely different. Moreover most of the available animal models are made in mice which allows us to mimic some aspects of the particular disease but not replicate all of the symptoms. However thanks to the models we are able to look for the answers for a specific questions about genetic and disease what will be impossible to achieve without them.

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3. Genetic manipulation of animals

In biomedical research use of experimental animals have been known for decades to investigate aspects of physiology and biochemistry. Artificial manipulations have often been limited to examining the effect of altering the animal's environment or some aspects of its phenotype. Traditional genetic manipulation of animals involved selected breeding experiments or exposure to powerful chemical such as base analogues, intercalating agents or physical agents such as radioactivity were and still are widely used to produce force random mutation in the animal genomes. After that process animals with phenotypic differences were separated and their genome completely analyzed to determine the genetic modification involved with that specific phenotypic changes. Some animals especially *Drosophila* and mice have been particularly suitable to that kind of manipulation.

A new era in animal research became when genetically modify animals have being introduced. In 1976 Rudolf Jaenisch published [1] the first report of insertion of a SV40 virus genetic material into mouse embryos. From that moment it become possible to perform genetic alteration no longer randomly but in a direct and specific manner. Few years later in 1981 Gordon and Ruddle reported [2] birth of a mouse that had been genetically modified through microinjection of purified DNA into single-cell mouse embryos. It was the beginning of transgenesis and controlled genetic alteration in animal models. Since then the use of genetically modified animals in research have been increasing tenfold for each decade and diverse models are available such as transgenic, knockout and conditional knockout, knock-in, humanized and knockdown animals. This models have many advantages for research especially in two areas – gene function studies and animal models of diseases.

The genetic modification of organisms such as *Drosophila*, *C.elegans*, yeast, viruses or bacteria can provide useful information on the fundamental biological role of genes. Unfortunately using this species we will not get the answer for effects of gene modification on the development of organs or physiological processes. Mouse is the most widely used as an experimental model for that type of study and for modeling the genetics of human disease. It is difficult to make a current estimate of the total number of available mouse mutant lines, but there are more than 3000 [3].

Trangenetic animal is produce when foreign DNA is artificially put into the cells of an animal. The DNA molecule is called a **transgene** and depend on our scientific questions what we would like to address it may contain one or many genes. Stabile transmission of a foreign DNA in the germline of transgenic animal is possible when we insert a transgene into a fertilized oocyte or cells from the early embryo. Many transgenetic animals especially mice have been created and the technology of transgenesis and its application is under constant developing. Moreover the ability to performed specific changes in a predetermined gene (gene targeting) has permitted to design of many new animal models of human disease.

There are several approaches that are routinely used for manipulating the mouse genome and generating genetically modified animal models. It is necessary to modify the DNA of germline cells that this modification become heritable. Certain cell lines were considered to be the optimal targets for introducing foreign DNA for example fertilized oocyte, very early stage embryos or embryonic stem cells (ES). To create an embryo with permanently altered genome and the foreign DNA will be permanently and irreversible incorporated into the genome of a host cells at least 3 steps have to be done:

1) *In vitro* gene construct have to be created
2) The exogenous DNA have to reach the nucleus of the host cell
3) DNA have to stabile integrate into host genome and have to be passed without any alteration to the next generation

Gene construct

It is possible to create different gene construct that can vary in complexity according to the insert. Another features which we can choose is the manner how the desire DNA will integrated into a host genome. It can be inserted into a genome of the animal in two ways – randomly which is called **trangenesis by addition**, or designed to be inserted at a specific targeted site into the correct position of a determined chromosome called **transgenesis by homologous recombination** . In both cases the structure of the gene construct have to be carefully planned and consist the structures to control gene expression, such as: a promoter, sides of transcription initiation and termination as well as polyadenylation. All of this is very important to avoid problems of uncontrolled expression in the host cell. The different

strategies can be used to insert DNA into the cells and they are presented in Table 7 (based on [4]).

Table 7. Methods used to insert exogenous DNA penetrate to host cell

Method	Description
Calcium precipitation	• DNA is precipitated with calcium salt, that kind of material is incorporated into endosomes and released into the cytoplasm and afterwards enters the nucleus • Method is quite simple but very inefficient, much of the DNA is degraded in endosome but its work for some cell culture types
Electroporation	• Cells are mixed with DNA in solution and exposed to a sudden, powerful electric current, which opens pores in the plasma membrane allowing entry of the DNA • Widely used, very efficient physical method which can be applied to most cell types
Lipid micelles	• Method based on the formation of complexes involving cationic lipid molecules of DNA, which protects against the nucleases • Applicable to most cell types, simple but less efficient than electroporation
Microinjection	• DNA in the solution is physically injected into the cell nucleus • It is widely used method in transgenesis for gene addition and is considered 100% efficient to carry the DNA into the nucleus of somatic cells and widely used for insertion of gene vectors into fertilized oocytes with success rate 4-8% of animals born with the transgene integrated into the host genome • It requires specialized equipment and highly skilled individuals to performed microinjection which is the main disadvantage
Viral vectors	• The newest method based on integration of the DNA of interest into a genome of a virus that is used to infect cell • Viruses used in this technique - SV40 (Simian virus 40), retroviruses, lentiviruses • High rates of efficiency of expression

The most widely used method to obtain transgenic mice is pronuclear microinjection (Fig.15). To obtain transgenic mice by this route females are superovulated, mated to fertile males and sacrificed the next day. From excised oviducts fertilized oocytes are recovered. In the next step foreign DNA is microinjected using a micromanipulator into the pronucleus of individual oocytes. Oocytes prepared in that manner are reimplanted into the oviducts of foster females and allowed to develop into mature animals. During the procedure transgene randomly integrates into chromosomal DNA usually at a single site. As a result of

chromosomal integration the transgenes can be passed on to subsequent generations in mendelian fashion [5].

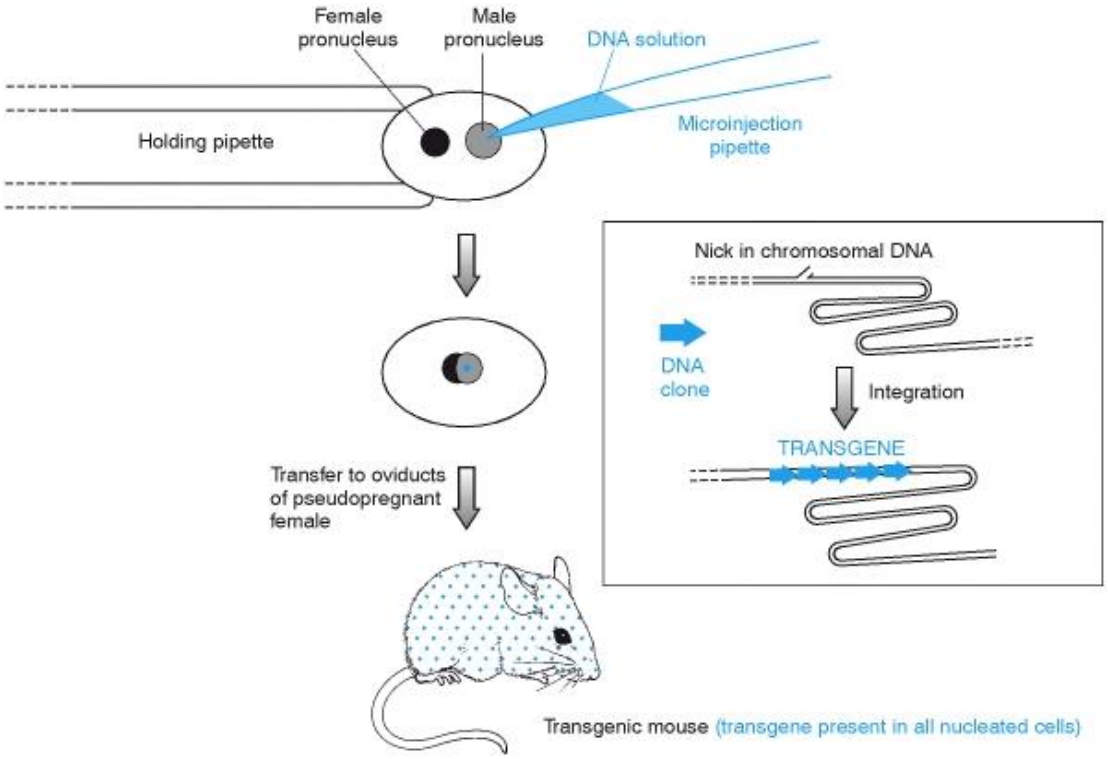

Fig. 15. Construction of transgenic mice by pronuclear microinjection (Adapted from [6]).

Alternative to that approach is transfer of the foreign DNA initially into cultured embryonic stem cells (ES). Mouse ES cells are produced from 3.5-4.5 day postcoitum embryos and arise from the inner cell mass of the blastocyst from a suitable mouse strain (129). Such prepared cells can be genetically modified while in culture and then injected into isolated blastocysts of another mouse strain (e.g. C57B10/J which has a black coat color that is recessive the agouti color of the 129 strain). In the next step that prepared blastocyst is implanted into a pseudopregnant foster mother. The offspring is a chimera containing two populations of cells, but when it is backcross can produce mice that are heterozygous for the genetic modification. Interbreeding of heterozygous mutants generates homozygotes (Fig.16).

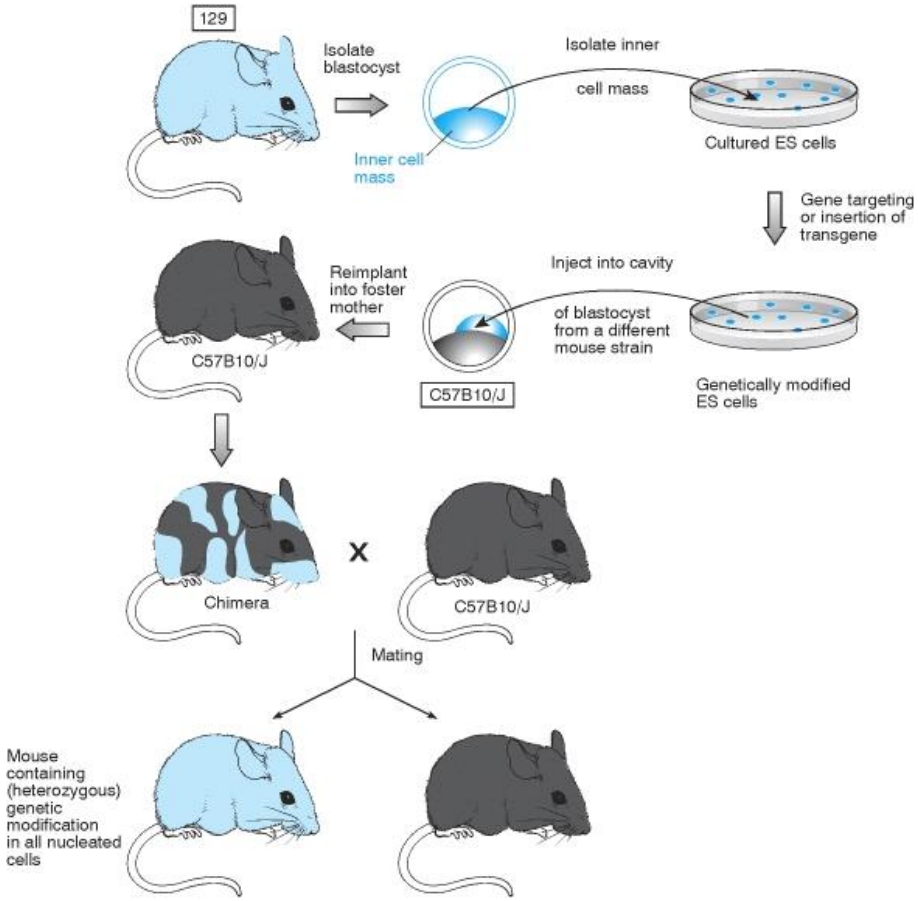

Fig. 16. Genetically modified ES cells as a rout for creating transgenetic animals (Adapted from [6]).

Integration of foreign DNA into the host genome occurs with a different efficiency depend on the method. It is believed that integration process occurs as a consequence of errors made when the DNA repair machinery of the host cell accidently incorporates exogenous DNA molecule [7]. DNA molecule break down randomly, integration of transfected DNA typically also occurs in random location in the genome. Incorporated exogenous DNA could potentially altered the expression of genes of the host but the probability is rare. Although when the integration events occasionally alters endogenous gene expression (**insertional mutation**) producing a recognizable phenotype. That kind of situation we called *in vivo* mutagenesis. The opposite approach is **gene targeting** in which the mutation is introduced into a preselected endogenous gene resulting that all cells contain a mutation at the desired locus [8].

For many applications it is desirable to control the expression of a transgene which can be achieved with a tissue specific or inducible promoter. Using molecular biology

techniques we are able to create structures which are called **transgenesis vectors**. This structures are composed of nucleic acid and derived from viruses, prokaryotic or eukaryotic plasmids that have an ability to carry gene of interest into the host cell nucleus and integrated it into the genome. The functional vector should contain:

1. Promoter and upstream regulatory sequences – region that determine the conditions of exogenous gene expression as well as location, promoters can be inducible which means that their activation can be mediated by drug or by the physiological state of the animals
2. Coding sequence – genetic information for the mRNA to be formed, it contains initiation codon (ATG) as well as stop codon (TAA, TGA, TAG)
3. Termination/polyA signaling sequence – addition of repetition of adenosine at the end of the mRNA provides stability of the molecule and allows its translation

3.1. Types of genetically modified animals

Transgenic animals

That type of modified animals possess in their genome exogenous gene which was artificially inserted and stably incorporated into the genome of every cell of the organism and can be transmitted to their offspring. The first described transgenic mouse had inserted sequence of the Maloney leukemia virus [1]. The protocol to obtain such animals are very different but in general proper construct of the gene is insert to the fertilized egg cell which allows for a random integration of the exogenous gene into the genome of the animal. The animal generated from this egg cell is called **transgenic founder** and is heterozygous for the transgene. Transgenic models are widely used in different types of research. We can evaluate in vivo the effect of the inserted gene during the developmental stages as well as in progress of diseases, studies of mutations, search for therapies.

Knock-out mice

This type of models are widely used to study gene function through the phenotype presented due to allelic deletion. The knockout model is obtained by target insertion that is performed through the mechanism of homologous recombination and it is not a very efficient process [9]. Homologous recombination technique allows the insertion of a

determined transgene into a specific locus at the desired part of the chromosome. The most common way to delete exons is by the creation of targeted construct, plasmid must contain a homologous part of the start of the gene (“left arm”) and a part obtained along the gene (“right arm”) [10]. Between that parts of the construct exon must be situated, that is important for the functionality of the protein. When that kind of vector will be introduced to the host cell these homologous regions will pair with corresponding sequences in the gene of interest. There is a dependence that the longer the arms the higher probability that the pairing will occur. The combination of both arms should be about 6 and 10 kb [11]. As a result of this pairing and a double recombination one in each arm is replaced by transgene containing the gene sequence to be introduced. Another important element which the construct may possess is a reference sequence of a positive selection gene i.e. *NeoR*. The process of double recombination is performed in ES cell culture and there are different protocols for cultivation and maintains of these cells [12]. When the ES cells are selected can be transferred by micromanipulation into the interior of murine embryos at about 3-4 days of age. The modified cells will attach to the normal one and will contribute to the formation of all tissues of the adult mouse which creates chimeric mouse (Fig.16). There are two big projects which aim to obtain strains with individual deletions for all genes as well as mutation known to be related to syndromes – KOMP (Knock-Out Mouse Project) and EUCOMM (European Conditional Mouse Mutagenesis).

Conditional knock-out

Deletion of genes which are essential for development may result in embryonic lethality thus mice die in the uterus making difficult to study events that occur after birth and impossible to study function of this genes in adult animals. One possible strategy which allows to avoid problems with embryonic lethality is conditional deletion system. This permit gene deletion under specific conditions. The main strategy is known as Cre-Lox system [9]. The Cre enzyme is a recombinase present in the P1 bacteriophage and it is responsible for mediation of DNA recombination of regions flanked by sequences loxP [13]. The loxP is a 34 bp sequence that can have a different orientation. When loxP flanks are in consensus the region of DNA between them is excited, but when the flanking sites are in opposite positions enzyme Cre reversing it. That kind of situation is possible to obtain in ES cells. The conditional control is based on the Cre enzyme, which may have its expression controlled by

the promoter and can be inherited form another transgenic animal. The promoter that regulates the expression of *Cre* gene will control the deletion pattern of the target gene which can be organ or cell type specific. The promoter may be regulated by drugs, physiological state of the animal or cell type.

The promoters what can be used for different conditions [9]:

1. Cell specific promoter – Cre recombinase will be express only in a determine cell or group of cells i.e. aP2 promoter allows to delete gene of interest only in adipocytes, αMHC promoter for deletion in cardiomyocytes, ALB for hepatocytes, Nestin for neurons, K5 for epithelial cells of the skin, CD19 for B lymphocytes, Cola1 for osteoblasts
2. Temporal promoter – regulatory sequence for gene expression only at specific period or physiological state i.e. CaMKII allows to delete gene in nurons only after birth
3. Inducible promoter – regulatory sequences which are activated or deactivated by exogenous molecules. The most wildly used is the TET operon system of *E.Coli* [14]. With this strategy tet-Cre animals are crossed with loxP animals, and homozygous offspring at the determined time of development are receiving tetracycline or its analogue, which cause excise the gene of interest

Knock-in mice

This technique allows to introduce new genetic elements into specific site. To achieve this after the codon stop sequence of the studied gene an entry site of the ribosome is added (IRES – internal ribosome entry site), followed by the cDNA sequence of the reporter gene. IRES sequence permits a single molecule of eukaryotic mRNA to code two different proteins [15]. The most commonly used reporters are enzyme β-galactosidase and GFP (green fluorescent protein)[16]. This technique can be used to study the expression of molecules, identifying cells that express and modulate expression. That type of animals also are used as a models of different types of disorders [4].

Mice with the fragments of chromosomes

It is possible to create artificial chromosomes such as PACs, YACs, and BACs. With this constructs it is possible to introduce to the host cells sequences with hundreds of kb

originated from human genome. This technique allows to obtain genetically modified mouse with human genes. This humanized mice are useful for the study of the expression of factors related to the immune system, identify genes related to human diseases as well as create new disease models. Another important use of these animals is the ability to produce human antibodies that can be injected into the patients without the possibility of recognition and rejection [17].

Knock-down mice

Constitutive or conditional knock-down of genes is performed using RNA interference techniques (RNAi). Long molecules of double-stranded RNA (dsRNA) can be processed into small interfering RNA (siRNA) with the action of ribonyclease Dicer. The complex called RISC (RNA-induced silencing complex) recognizes and cleaves mRNA complementary to antisense siRNA. mRNA which has to be cleaved is rapidly degraded [18]. In mammals dsRNA longer than 30 bp provoke global response of protein synthesis inhibition and non-specific degradation of mRNA. It is possible to create small synthetic dsRNAs which will serve as a triggers for specific breaks of mRNA in animal cells. To make it possible, and to create knock-down animal we have to design specific vector. Such a construct have to contain a sequence that encodes an RNA transcript complementary to the target mRNA as well as hairpin region. Forming of shRNA is a key feature for Dicer enzyme to act and form siRNA. This construct should be stabile integrated into the genome that allows it to pass through generations. The big different between knock-out and knock-down animals is that in the first model the target protein is completely absent while in the second one we achieve decries in protein quantity up to 90%. Knock-out animals have the sequence responsible for mRNA production deleted form the genome, while in knock-down animals the interference process acts during post-transcriptional phase [19]. In some cases it is not necessary to permanently knock-down the target protein, and for that purposes we can obtain the knock-down for specific periods. It can be done when we associate the expression of RNAi with an inducible promoter controlled by doxycycline (Tet-On inducible expression system)[20].

After the creation of the genetically modified animal and before it will serve as a proper model for human genetic and disease study it have to be well characterized. This type of characterization is called phenotyping, and can be divided into three steps [4]:

1. Clinical and morphological characterization – morphological characteristic and evaluation of all vital parameters (i.e. hart and respiratory rate, blood pressure, behavioral analysis, profile of serum molecules, histological analysis of organs, monitoring of growth and development
2. Molecular characterization – evaluation of DNA, mRNA and proteins, identify the region of vector integration into the genome as well as the number of copies inserted
3. Pathological characterization

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4. Treatment of genetic disorders

Once the people suffering from the genetic disorders are completely investigated to find out the exact disease they are affected with, so that the patients are correctly diagnosed, they have to be set into the most proper treatment. The cure of genetic diseases is a method of combating, ameliorating, or preventing a disease caused by abnormalities in genes or chromosomes. It is being developed over the past forty years. Knowledge of the human genome sequence and the catalogue of human genes, together with advances in molecular biology, protein engineering, and bioengineering, have an enormous impact on the treatment of genetic disorders. It is obvious that the understanding of genetic disorder at a molecular level is the foundation of reasonable therapy. In the following chapter, we review approaches of therapies for genetic diseases, from those well established and being successful implemented, to new strategies that are still in the exploration phase and seem likely to be employed in the future.

4.1. The current state of treatment of genetic disease

Genetic disorders can be treated at various stages and at many levels. Below we describe the foundation used or proposed for treatment at each of these levels. These include: from classical approaches to advanced forms of treatment, for preventive to symptomatic therapies [1-5]. The prophylactic treatment that in which the aim is to prevent the occurrence of the disease offers the possibility to identify a predisposition of an individual to a particular sickness, even before clinical symptoms are manifest, or to predict the probability of occurrence of the disease. When a genetic predisposition has been identified, all that is possible in many cases is to predict the probability of disease manifestation. On the other hand, predictive diagnosis can provide the opportunity to identify a disease at an early stage and start a therapy or take preventive measures. Techniques being used in preventive therapy to screen for genetic diseases include: (*i*) preimplantation diagnoses (i.e. testing for diseases on embryos acquired during *in vitro* fertilization), (*ii*) prenatal analyses (i.e. testing for disease on an embryo or in a fetus before it is born), (*iii*) newborn screening (i.e. used just after birth to identify genetic disorders that can be treated early in life), (*iv*) carrier testing (i.e. used to identify people who carry one copy of a gene mutation that, when present in two copies causes a genetic disorder), and (*v*) presymptomatic testing for predicting adult-onset disorders or estimating the risk of developing adult-onset disease [6-9]. Unfortunately, predictive

genetic diagnosis may also entail certain dangers as a result of wrong, contradictory or undesirable information. Therefore the performance of predictive tests should ever be subject to special conditions.

On the other hand, once symptoms of genetic disorders occur, the symptomatic therapies aimed at assisting with the handling of diseases begin. All currently existing symptomatic treatments are limited, able to partially alleviate, rather than halt disease signs. They are often called supporting treatments, as they are mainly directed to sustaining the strength of the patient. Symptomatic treatment is also referred to palliative treatment, designed to relieve pain and distress with no attempt to cure.

Although still most genetic disorders cannot be treated, various developmental studies are in progress towards safe and efficient pharmaceutical-, cell- and genetic-based therapies. Some of these therapeutic approaches are already moved into clinical trials, while others need further detailed investigations. The new prospects offer hope for significant delaying and possibly halting these debilitating diseases, which are genetic disorders.

4.2. Therapeutic strategies

The objective in treating genetic disease is to eliminate or ameliorate the effects of the disorder, basically in two ways: either with classical approaches, belonging to the pre-genetic medicine era, where also some sophisticated molecular treatments, such as proteomic therapy fits in, or with advanced therapies derived from genetic medicine.

4.2.1. Classical forms of treatment

It is obvious that environmental interventions, such as medications, lifestyle or diet modifications, may have a huge impact on the management of genetically determined diseases. These known as the classical strategies aim basically to prevent organ damage from excessive amounts of metabolites generated by the metabolic defect or add substances that are not synthesized [2]. Broadly speaking they focused on metabolic manipulation and protein augmentation therapy. Among the metabolic manipulations the simplest form is diet modification, which include: avoidance, dietary restriction, replacement, diversion, inhibition, and depletion (Table 8) [1].

Table 8. Treatment of genetic disease by metabolic manipulation.

Type of metabolic manipulation	Substance or technique	Disease
Avoidance	Antimalarial drugs	Glucose-6-phosphate dehydrogenase deficiency
Dietary restriction	Phenylalanine Galactose Lactose	Phenylketonuria Galactosemia Hypolactasia
Replacement	Thyroxine Biotin Biopterin	Congenital hypothyroidism Biotinidase deficiency Tetrahydrobiopterine deficiency
Diversion	Sodium benzoate Oral resins that bind bile acids Drugs that block the intestinal absorption of cholesterol	Urea cycle disorders Familial hypercholesterolemia heterozygotes
Inhibition	Statins Allopurinol	Familial hypercholesterolemia heterozygotes Gout
Depletion	LDL apheresis (direct removal of LDL from plasma) Penicillamine Cysteine Phlebotomy	Familial hypercholesterolemia homozygotes Wilson's disease Cystinuria Hemochromatosis

Dietary restriction is one of the oldest and most effective approaches of managing genetic disease. The restrictive intake of specific dietary component to diminish the metabolic load on defective metabolic step, usually requires lifelong compliance with a severe restricted and even artificial diet. Currently, diseases involving more than tens loci are managed in this way (e.g. lactose restriction for lactase deficiency; phenylalanine restriction to treat

phenylketonuria). Furthermore, the oral replacement of essential metabolites, cofactors, or hormones whose syntheses are affected due to a genetic disease is often simple in application (e.g. biopterin in tetrahydrobiopterin deficiency, biotin in biotinidase deficiency). Next, diversion therapy, which is binding of metabolites blocked up-stream of the metabolic hindrance and the enhanced use of alternative metabolic pathways to reduce the concentration of a harmful metabolite, is another strategy of treatment of genetic diseases (e.g. the urea cycle disorder, where ammonia cannot be removed because of a genetic defect of a urea cycle enzyme, in this the administration of large quantities of sodium benzoate diverts ammonia to glycine synthesis, which results in hippurate formation being subsequently excreted in the urine). Also, the pharmacological inhibition of the activity of the malfunctioning enzyme is sometimes used to modify the metabolic abnormalities of genetic errors (e.g. the use of allopurinol to inhibit xanthine oxidase and prevent uric acid being formed in gout). Finally, the use of chelating agents to treat the genetic diseases is reported. Characterized by the accumulation of a harmful compound disorders are sometimes treated by direct removal of the compound from the body (e.g. the use of penicillamine for the elimination of cooper in Wilson's disease, cysteine in cystinuria, phlebotomy to alleviate the iron accumulation in hemochromatosis).

4.2.2. Surgical approaches - organ replacement therapy

Even if more invasive, transplantation has long been used and continues to be utilized to treat certain genetic diseases that affect particular organs [1,10]. Although complementary rather than competing with other treatment approaches of genetic illnesses, organ replacement handling remains a viable therapy that continues to be used extensively to this day. It is the 'ultimate genetic medicine', as apart from the relevant somatic stem cell and differentiated cells treatment, it involves replacement of the organ that is malfunctioning secondary to the abnormal phenotype. Unless the organ donor and the organ recipient are monozygotic twins, the genetic material sequence of the donor will be different from that of the recipient, which unfortunately hinders the procedure. Organs transplantation for genetic disorders includes replacing the liver, kidney, lung and heart. Organ transplantation has been used successfully to treat patients with various deficiencies (such as liver transplantation in tyrosinemia, and kidney replacement in Fabry disease), as the genetic defect is specifically expressed in the affected organ. This forms of treatment is limited by the nature of the surgical intervention involved and by a substantial lack of donor organs. What is more, immunosuppressive handling is required both for the transplantation of cells and organs. Ultimately, future

alternatives could include generation of a new organ. Various new technologies, including xenotransplantation (i.e. transplantation of human organs developed in animals), and organogenesis (i.e. the *de novo* development of organs), all have potential for replacing or augmenting organ function [11]. However, biological and immunologic obstacles may delay application of these technologies [12]. In another approach, the genetic medicine can be designed to regenerate a diseased organ, either by re-engineering tissues by expressing embryonic key genes that induce organ development or, in the case of stem cell therapy, to generate normal tissues. Details regarding these strategies are discussed in the next sections.

4.2.3 Advanced methods - the molecular treatment of genetic disease

The molecular treatment of genetic disease include also approach based on pre-genetic medicine, which is the proteomic therapy. The concept underlying this strategy is that transfer of proteins, peptides, etc., or acting of chemical molecules on proteins will result in modified phenotype for therapeutic approaches.

Other molecular treatment strategies for genetic disorders include transfer of genetic material with the use of somatic stem cells (SSCs) and embryonic stem cells (ESCs) (for entire genome transfer), gene transfer (for single gene transfer), and RNA modification (for transfer of coding sequences). For each of these strategies, the fundamental approach is to modify the gene or its expression in cell, tissue and organ of the affected individual.

4.2.3.1. Treatment at the level of the protein - proteomic therapy

Enhancement of mutant protein function with small molecule therapy is being used as treatment of genetic disorders at the level of the protein [1]. Small molecules, synthetic or natural, such as vitamins, non-peptide hormones, and most drugs are class of compounds with molecular weights in the few hundreds to thousands.

Vitamin-responsive inborn errors of metabolism is one of treatments of genetic disorders at the level of the protein. The biochemical defects of a number of metabolic diseases may in fact respond to the administration of large amounts of the vitamin cofactor of the enzyme impaired by the mutation (e.g. the use of high doses of pyridoxine (vitamin B6) in homocystinuria). Furthermore, the small molecules that modify the conformation of misfolded mutant proteins were also identified as efficient in genetic disorder treatment. The administration of such molecules may be used to overcome a folding defect, as they enable mutant proteins to move and/or function normally, or otherwise trick chaperones and other specific control systems to accept the misfolded protein (e.g. impairment of the ΔF508 protein

binding in the endoplasmic reticulum by the calcium-dependent chaperone as a result of the administration of curcumin in cystic fibrosis). In another approach, the small molecule therapy is associated with skipping over mutant stop codons (e.g. in cystic fibrosis, aminoglycoside antibiotics convert the stop codon to an amino acid, a substitution that generates a peptide with nearly normal properties). Also, the concept of protein augmentation therapy, i.e. purification of the missing, defective or depleted protein, followed by its delivering to the patient is successfully implemented in treatment of genetic disorders, including various membrane transport disorders (cystic fibrosis), coagulation disorders (hemophilia A, hemophilia B, and Von Willebrand disease), emphysema (ά1-antitrypsin deficiency), immune deficiency (severe combined immune deficiency), endocrine disorders (growth hormone deficiency, congenital leptin deficiency, and congenital neurogenic diabetes insipidus), and lysosomal storage disorders (LSDs) (Gaucher's disease type I, Fabry disease, mucopolysaccharidosis I, mucopolysaccharidosis II, mucopolysaccharidosis VI, and Pompe's disease). In some cases, the success of protein augmentation depends on how well the protein is delivered to the organ in which its function is required. The brain is a particularly difficult organ to target, because the access of proteins is limited by a membrane structure called the blood-brain barrier (BBB). The BBB inhibits the transfer of proteins and other chemicals from the bloodstream into the brain. A similar problem occurs for the eye treatment. Though, therapeutic agents can be transported directly to the eye, while is not possible with the brain. Protein augmentation therapy is most applicable to treating disorders in which the defective protein functions in the extracellular space. The avoidance or inhibition of bleeding episodes in patients with hemophilia A by the infusion of plasma fractions enriched for factor VIII is the prime example here. Also, replacement of alpha-1-antitrypsin in alpha-1-antitrypsin deficiency is described as therapy based on replacing an extracellular protein. On the other hand, the protein augmentation therapy involving an intracellular protein replacement can be effective only if there is a mechanism to import the protein into impaired cell compartments. Such extracellular replacement of an intracellular protein is characteristic for lysosomal storage diseases including mucopolysaccharidoses, Sachs disease, for adenosine deaminase deficiency, while replacement of an intracellular protein by cell targeting is known for modified glucocerebrosidase in Gaucher disease. The concept of this treatment is based on principle at which the therapeutic enzyme has to be transferred to its intracellular site by means of intramuscular or intravenous administration (e.g. to the lysosomes in LSDs). The efficiency of enzyme replacement therapy (ERT) was not very high when implemented, though it is significantly improved by use of the modified enzymes, being at the same time

less immunogenic as observed before. However, still other problems resulting from treatment of genetic disorders with protein augmentation therapy such as requirement of frequent, repeated administrations, cost, infections, maintaining venous access to administer the enzyme, and supply shortages of the therapeutic agents have to be faced.

4.2.3.2. Treatment at the level of the cell - cellular therapy

Cellular therapy acts on stem cells (SCs), either on somatic or embryonic. SCs are unspecialized cells that are defined by their capacity for self-renewal and the ability to differentiate into specialized cells along many lineages [13]. They naturally occur in two forms: (*i*) somatic stem cells (driven from various fetal and postnatal organs, and are multi- or unipotent), and (*ii*) embryonic stem cells (driven from the inner cell mass of embryos at the blastocyst or earlier morula stage, and are pluri- or totipotent, respectively) [14, 15].

Somatic stem cells can be found in specific tissues in the body, and are usually named on the basis of the organ from which they are driven. Basically, two sources of SSCs are described, which are hematopoietic and non-hematopoietic cells [16]. Hematopoietic stem cells (HSCs) can be found in adult bone marrow and blood and umbilical cord blood. These stem cells are regularly used in standard therapies, as they make new blood cells. Adult bone marrow stem cells are utilized in bone marrow transplantation (BMT) in the clinic for more than 40 years as a means to replenish the body cells that can differentiate into all lymphoid and myeloid blood lineages (such as leukocyte refilling in patients suffering from leukemia). It is also worth mentioning that the purpose of a bone marrow stem cell transplant for children with genetic disorders is to provide special marrow-derived cells, which travel to various organs in the body including the liver (Kupffer cells), lungs (alveolar macrophages), skin (Langerhan's cells), spleen (macrophages), lymph nodes (lymphocytes and macrophages), and brain (microglia). Furthermore, peripheral blood stem cells can be collected from circulating blood and used to treat blood disorders, leukemia and other cancers. A particularly promising source of stem cells for treating various disorders is umbilical cord blood. In recent years, the blood in the cord has been found to be a rich source of SCs that are less susceptible to rejection from a transplant recipient's body than cells or tissues transplanted from another individual. In sum, HSC transplantation from individuals that express the normal gene has been used to treat many genetic errors including lysosomal storage disorders, immunodeficiences, hemoglobinopathies and leukodystrophies.

Besides to HSCs, the non-hematopoietic stem cells (non-HSCs) have also been identified in the brain, skin/hair, heart, liver, gut, pancreas and skeletal muscle, known respectively as neural stem cells, skin stem cells, intestinal stem cells, etc.

A second population, called bone marrow stromal stem cells (also called mesenchymal stem cells), were also discovered. These non-HSCs are small amount of the stromal cell population in the bone marrow, and can create bone, cartilage, fat, cells that support the formation of blood, and fibrous connective tissue [17].

Although so far there are no approved treatments using embryonic stem cells, many attempts have been made in order to implement them, inter alia, to the cure of genetic disorders [18]. Differentiation of ESCs into usable cells while avoiding transplant rejection, also their ability to form tumors are just a few of the hurdles that embryonic stem cell researchers still face. Also, technical difficulties in obtaining and culturing these cells, as well as ethical concerns regarding the use of human embryos as a source of stem cells have hindered the clinical usage of human ESCs (hESCs). The use of human embryonic stem cells as models for human genetic disorders has started to address this issue. This has been done either by genetically manipulating the cells, or more recently by deriving diseased cell lines identified by prenatal genetic diagnosis. These studies are however still contravention as they are important issues of human being, the foundation of which is to guarantee the freedom to live, where there are no question regarding whether or not a potential human life at the embryonic stage should be granted the moral status of a human being.

In addition to stem cells that are isolated from the body, investigators have found an approach to produce stem cells by using various reprogramming techniques in the laboratory. Two such reprogramming methods are: (*i*) the creation of induced pluripotent stem cells (iPSCs) [19], and (*ii*) the use of somatic cell nuclear transfer (SCNT) [20]. The ability to convert somatic cells into pluripotent stem cells was one of the most exciting breakthroughs in stem cell research that occurred recently, and which has been quickly reproduced in labs across the world [19, 21-24]. Scientists have identified a set of four genes (*OCT4/POU5F1*, *SOX2*, *KLF4*, and *c-MYC/MYC*) that, when expressed at the same time, can transform skin cells (dermal fibroblasts) derived from an adult human into iPS cells. It is important to notice that the induced pluripotent stem cells are phenotypically indistinguishable from human embryonic stem cells in terms of their gene expression, cell surface markers, and cellular morphology. Similar to the hESCs, the induced pluripotent stem cells are immortal,

pluripotent, and capable of expressing genes corresponding to all three embryonic cell layers (endoderm, ectoderm, and mesoderm) when induced to differentiate. iPSCs may also be an extremely powerful and invaluable tool for making stem cell lines as disease models. Understanding the underlying mechanisms associated with disease, among them with genetic disorders, is possible by implementation of iPS cells. Lately, a team of researchers applied this technique and developed a panel of human disease-specific iPS cell lines, by collecting skin cells and bone marrow-derived mesenchymal cells from patients with one of ten various disorders, including immunodeficiency (ADA-SCID), Shwachman-Bodian-Diamond syndrome (SBDS), Gaucher's disease (GD) type III, Duchenne muscular dystrophy (DMD), Becker muscular dystrophy (BMD), Parkinson's disease (PD), Huntington's disease (HD), juvenile-onset type 1 diabetes mellitus (JDM), Down syndrome/trisomy 21 (DS), and Lesch-Nyhan syndrome. Like the original iPS cell lines, the disease-specific iPS cell lines were immortal, pluripotent, and capable to express genes characteristic of all three embryonic germ cell layers when induced to differentiate [25].

In 2009, it was demonstrated that creation of iPS cells is possible without any genetic modification of the adult cells . Pluripotency of the cells treated repeatedly with certain proteins guided into the cells via poly-arginine anchors was obtained, and these iPSC cells were given the acronym piPSCs (protein-induced pluripotent stem cells) [26]. In the same year, a Canadian-Scottish research team announced a method to create iPS cells using the piggyBac transposition, instead of utilization of retroviruses, hereby increasing the potential use of iPS cells in human diseases [27].

iPSCs are an important advance in stem cell research, as they may allow scientists to receive pluripotent stem cells demonstrating the potential for therapeutic use. As already mentioned, they are also an extremely powerful tool for creating stem cell lines as disease models, without the controversial implementation of embryos. Therefore, the promise of somatic cell-derived iPS cell lines was warmly welcomed by both opponents and proponents of human stem cell research. Although, additional questions about the ethical status of iPS cells have been raised recently and require consideration. Namely, it has been suggested that like human embryonic stem cells, iPS cells can also generate germ cells, ova and sperm cells, what can make the hESCs and iPSCs not be as ethically distinct as first suggested.

A somatic cell nuclear transfer is another way to create stem cells [20]. It uses a human egg (oocyte), in which the original nucleus is replaced with the nuclear DNA from a donor cell.

The reprogrammed oocyte with the entire complement of DNA is stimulated in the laboratory and becomes a blastocyst with the same genetic make-up as the donor. At this stage hES cells can be extracted and used to create a cell line culture, enabling investigators to create genetically identical tissue for tissue replacement (i.e. in autologous transplantation), and disease-specific cell line models. SCNT is a type of reprogramming technique that is also used in 'cloning' technology. Therefore it is often referred to 'research or therapeutic cloning'. There has been significant controversy about this method, in fact justified, and concerning the use of this method for reproductive cloning.

4.2.3.3. Treatment at the level of the gene - genomic and transcriptomic therapies

Of all of the therapeutic options for treating of genetic disorders, genomic and transcriptomic therapies seem to be the most obvious and prominent. Genomic therapy acts on genes, while transcriptomic on transcripts. The principle sounds simple enough: missing or defective genes need to be replaced by normal genes that can code for functional proteins. However, despite the many animal models of genetic disorders that have been successfully treated at the level of a gene, correcting human genetic disorders in this way has been difficult.

For genetic diseases, gene therapy offers the potential to introduce and express cloned genes in the cells of patients in order to overcome the disorder. Basically, there are two strategies of gene transfer for genetic disorders: *ex vivo* and *in vivo* gene transfer [1, 5].

Gene transfer ex vivo

Generally speaking, e*x vivo* gene therapy means that the cells are modified outside the body before being implanted into the patients. In detail, cells to be corrected are drawn from the affected individual and subjected to genetic alteration by transfer of cloned genes. The successfully transformed cells grown in culture are then introduced for replacement into the patient. This approach is only applicable to disorders in which the relevant cells can be removed from the body, modified genetically and returned to the patient where they will engraft and survive for a long time (e.g. bone marrow cells, skin cells, etc.). Another *ex vivo* approach being used for the applications for genetic diseases includes the implementation of corrected cells as sources of a secreted proteins (e.g. in hemophilia A treatment the *F8* gene is transferred to analogous fibroblasts).

Gene transfer in vivo

In vivo gene therapy involves the genetic modification of cells by introduction of the cloned gene directly into cells of the patient. The 'currier' containing the therapeutic gene is administered either directly to the organ of interest (*in situ*) or into blood vessels that feed the specific organ (*in vivo*). This approach may be the only possible option where relevant cells cannot be cultured *in vitro*, such as brain cells. Since there is no way of selecting and amplifying genetically modified cells, the success of this therapy is highly dependent on the overall performance of gene transfer and expression. Although a number of gene transfer methods have been developed, most of them are too inefficient for real use in clinics. Basically, available methods include chemical (polymers, dendrimers, inorganic nanoparticles, etc.), physical (electroporation, gene gun, ultrasound, hydrodynamic pressure, etc.), and fusion solutions for utilization of non-viral (receptor-mediated endocytosis) and viral gene-transfer vectors. There are five classes of gene-transfer vectors and single expression cassette model that are used to treat genetic disorders (Fig. 17).

Expression cassette

Usually, the gene is administered within an expression cassette that is composed of therapeutic transgene (in the form of cDNA or genomic DNA) flanked at the 5'end by the promotor and on the 3' side by a transcription stop and polyadenylation site (Fig. 17A). Efficient gene transfer with the use of expression cassette involves a number of complications, which relate to a relevant cell targeting, followed be entry into the nucleus where the therapeutic transgene has to be expressed. Though the principle sounds simple, it is challenging in practice in fact. To accomplish this, non-viral and viral-gene transfer vectors are used.

Liposome/micelle-based plasmid non-viral vectors

The non-viral vectors of plasmid DNA, naked or formulated with liposomes, consists of an origin of DNA replication and antibiotic resistance gene for production in bacteria (Fig. 17B). Naked DNA delivery is performed by a physical method, while delivery mediated by a chemical carrier such as cationic polymer and lipid is used to enhance the efficiency of transferring the plasmid to nucleus. There have been many attempts, some are still running, to correct genetic disorders with non-viral vectors. Several human trials carried out for example in hemophilia A or in cystic fibrosis have failed when using this strategy, as transgene expression tends to be transient and limited by degradation. Despite, non-viral gene delivery systems continue to be explored in certain situations, particularly where viral delivery remains

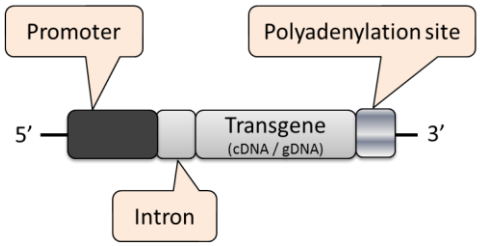
a problem or where repeat administrations increase the risk of an immune response to a viral antigen. Aerosolizing plasmid DNA, to enable delivery through inhalation (for example for treating the respiratory manifestations of cystic fibrosis), has also been accomplished [28].

Viral vectors - adenovirus, adeno-associated virus, retrovirus, lentivirus, pox viruses, alphaviruses, and herpes viruses

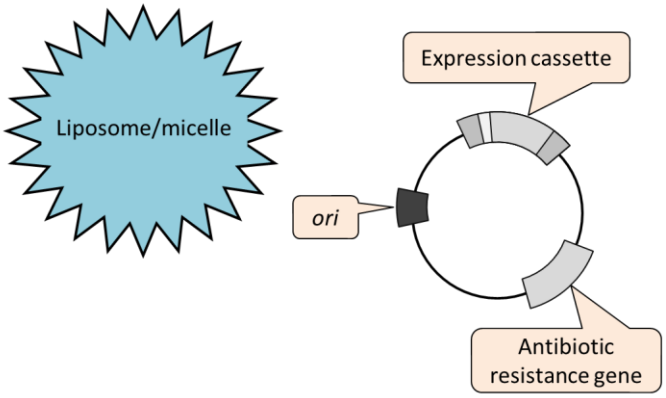
Viral vectors are a tool commonly used to deliver unmutated genetic material into target cells (Fig. 17C, D, E and F). This procedure can be performed inside a living organism (*in vivo*) or in cell culture (*in vitro*). Many gene therapy clinical trials rely on adenoviruses, adeno-associated viruses, retroviruses, lentivirus, pox viruses, alphaviruses, and herpes viruses. These viruses differ in how well they transfer genes to the cells they recognize and are able to infect, and whether they alter the cell's DNA permanently or temporarily. The most attracted viral vectors are the DNA-based adenovirus and adeno-associated virus vectors (AAV vectors), and the RNA-based retrovirus and lentivirus vectors. Adenovirus vector is the 'gold standard' of gene transfer vectors as it was the first gene transfer vector to be used *in vivo* to treat an hereditary disorder . Adenoviral dsDNA does not integrate into the genome and is not replicated during cell division, which limits the broader application of adenoviruses. On the contrary, adeno-associated viruses may incorporate its genome (ssDNA) into that of the host cell. These features make AAV a very attractive candidate for creating viral vectors for gene therapy. It has been shown that AAV vector-mediated gene transfer has corrected the abnormal phenotype of several animal models of genetic disorders, as well as in humans, including cystic fibrosis, muscular dystrophy, hemophilia B, Canavan disease and Batten disease. The drawback of using AAV vectors is its small cargo space available for delivery of a therapeutic gene. On the other hand, retroviruses are one of the mainstays of current gene therapy approaches. The recombinant retroviruses have in fact the ability to integrate into the genome of the target cell in a stable fashion. However, this feature also carriers the risk of insertional mutagenesis, which results in cancer. Lentiviruses, a subclass of retroviruses, have recently been adapted as gene delivery vehicles thanks to their ability to integrate into the genome of non-dividing cells. This unique feature of lentiviruses makes them special, as other retroviruses can infect only dividing cells. The viral genome (i.e. RNA) is reverse-transcribed when the virus enters the target cell to produce DNA, which is then inserted into the genome at a random position by the viral enzyme. Despite the fact that a huge number of laboratory successes with viral-mediated gene therapy is observed, this type of treatment continues to be in crisis owing to the lack of acceptable systems to deliver therapeutic genes to patients. The

further development of viral vectors as gene delivery tools is required to overcome serious complications associated with their use. The inserted virus-based vector can be perceived as a foreign invader by the immune system. As a consequence, the immune system releases antibodies to destroy the virus. The reaction of the immune system to the genetically modified virus could be very severe, often leading even to organ failure. In addition, the use of viruses as vectors poses a risk of viral spread in the organism resulting in other diseases such as cancer, alteration of the inserted virus into its original disease-causing form and genetic changes in the reproductive cells. As each approach of gene transfer has some limitations, there have been some hybrid methods developed that combine two or more techniques, such as mixing viral vectors with cationic lipids or hybridizing viruses.

A. Expression cassette



B. Liposome/micelle-based plasmid and nacked plasmid non-viral vectors



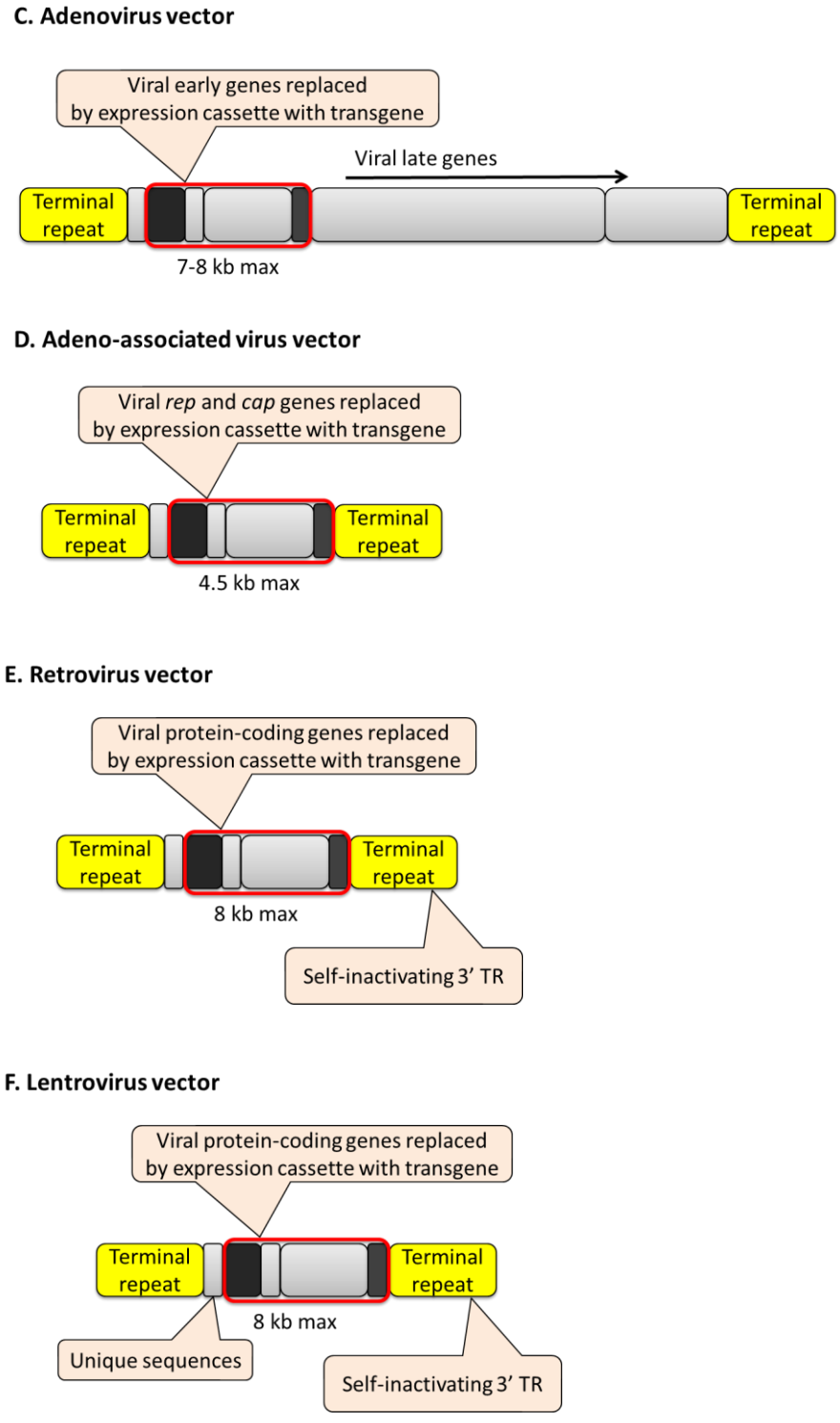


Fig. 17. Gene-transfer vectors used to treat genetic disorders.

On the genetic horizon, the modern-day equivalent of genomic therapy is likely to be the use of transcriptomic therapy, called also RNA-modification treatment [1, 5]. For individuals currying a dominant mutation, introduction of the corresponding wild-type gene is usually not sufficient to rescue the disease-associated phenotypes. Rather, in such a case to turn off the expression of mutant genes, suppressing transcript levels, or to inhibit the function of the mutant proteins their encodes is desired. To achieve this goal, investigators have turned their attention to RNA-based approaches, which target the RNA (either pre-mRNA or mRNA) transcribed from the dominant negative gene and effectively hinder expression of the mutant protein. RNA-based strategies for the treatment of disease are depicted in Fig. 18. They are five basic approaches to modifying RNA levels: antisense oligonucleotides (ASO), RNA interference (RNAi), *trans*-splicing, segmental *trans*-splicing, and ribozymes.

Antisense oligonucleotides

In ASO-mediated cleavage of mRNA, short single-stranded DNA (ssDNA) molecules, usually between 18 and 30 bases long, are designed to hybridize to specific regions (by complementarity) of targeted mRNA (Fig. 18A). The resulting DNA-RNA hybrid complex in the cell up-regulates expression of the intracellular enzyme ribonuclease H (RNase H), which cleaves RNA in the RNA-DNA hybrid molecules, leading to reduced expression of the protein that is encoded by the target.

RNA interference

RNAi strategy involves the use of small double-stranded RNA molecules (dsRNA, precursor of RNAi), typically 22 base pairs long, with 2-3 nucleotide 3’ overhangs, corresponding to a region of the target gene (Fig. 18B). The dsRNA is processed within the cell in such a way that it becomes part of an RNA-induced silencing complex (RISC) that recognizes and destroys the corresponding target mRNA.

Spliceosome-mediated trans-splicing

The third strategy, which is based on *trans*-splicing phenomenon, differs from both of the above ASOs and RNAi. Indeed, except for reduction of expression levels of targets, the spliceosome-mediated *trans*-splicing may also be utilized to modify the genetic repertoire at the pre-mRNA level to correct the phenotype. *Trans*-splicing is a gene-transfer approach that targets endogenous pre-mRNA containing an alteration within one of its exons (Fig. 18C). Therein, a gene-transfer vector is used for delivery of transgene that is required to replace the

exon carrying the disease-associated mutation with a wild-type copy of the exogenous exon. The transgene contains a hybridization domain, which is complementary to a region of the 5′ flanking intron between the donor and branchpoint sites for RNA splicing, followed by the splicing branchpoint, the splice acceptor site, the wild-type exon sequence, and the rest of the gene. Spliceosome-mediated *trans*-splicing results in corrected, wild-type copy of the mRNA and thus a corresponding wild-type protein.

Spliceosome-mediated segmental trans-splicing

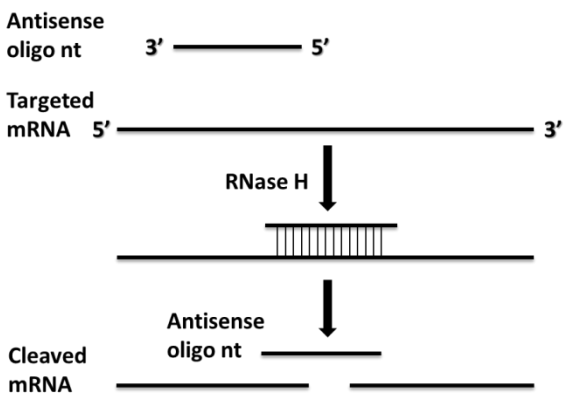
To avoid size limitations associated with gene-transfer methods that involve vectors, a new model, namely spliceosome-mediated segmental *trans*-splicing was established. In this approach, the gene is divided into two smaller pieces, which are delivered together using two separate gene-transfer vectors (Fig. 18D). The vector carrying the second half of the gene contains a hybridization domain complementary to an intron located at the 3′ end of the first half of the gene, analogous to that described above, i.e. for *trans*-splicing. Pre-mRNAs are then joined through *trans*-splicing to create an intact per-mRNA, and further to produce a mature mRNA encoding the full length of the wild-type protein of interest.

Ribozyme-mediated trans-splicing

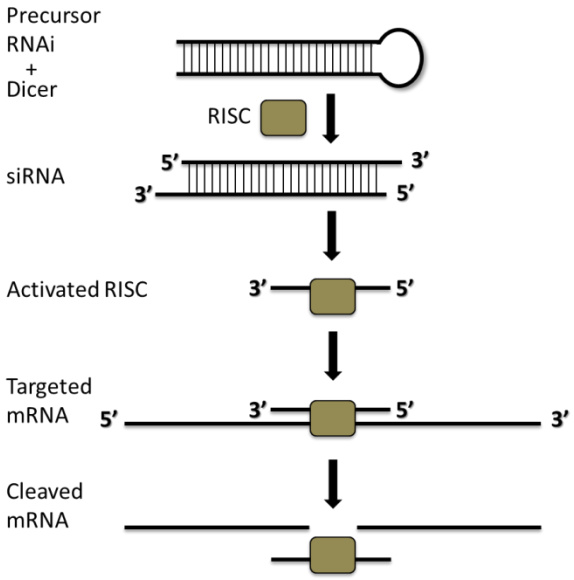
Ribozymes are RNA molecules with enzymatic activity that recognizes a particular mRNA sequences and cleaves them (Fig. 18E). The structure of ribozymes consists of two regions of antisense RNA, containing a hybridization domain followed by a ribozyme nucleolytic motif that recognizes a target mRNA and the corresponding wild-type gene sequence. Once expressed, the correct ribozyme binds to the target, cleaves the endogenous target molecule and replaces the defective exon with a correct sequence, leading to the formation of a wild-type copy of the mature mRNA.

Many of these RNA-based strategies have been established in recent years, still numerous questions regarding the cellular mechanisms involved in transcript targeting and problems related to the delivery of mRNA remain. Because the effective delivery is crucial, the breakthrough for RNA-modification therapies for treating of genetic disorders will depend on progresses in the development of gene-transfer vectors. In addition, a particular attention with respect to the specificity of mRNA-targeting approaches, to ensure that only the gene of interest is targeted must be paid.

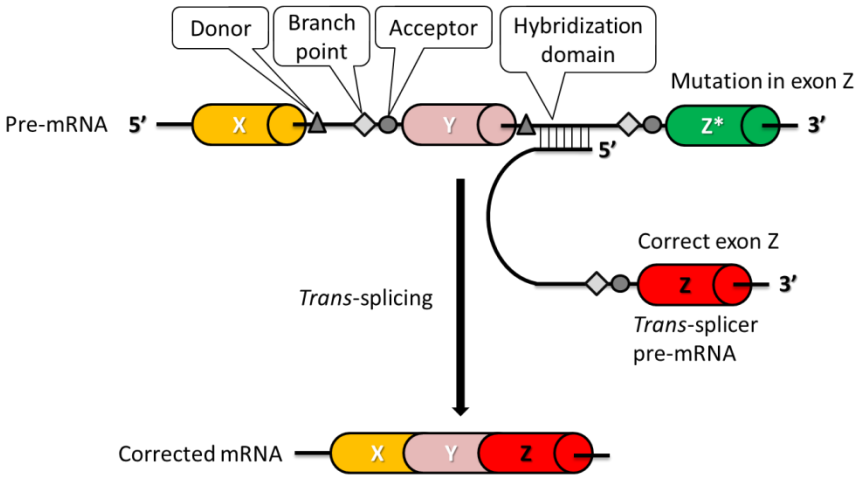
A. Antisense oligonucleotides



B. RNAi



C. *Trans*-splicing



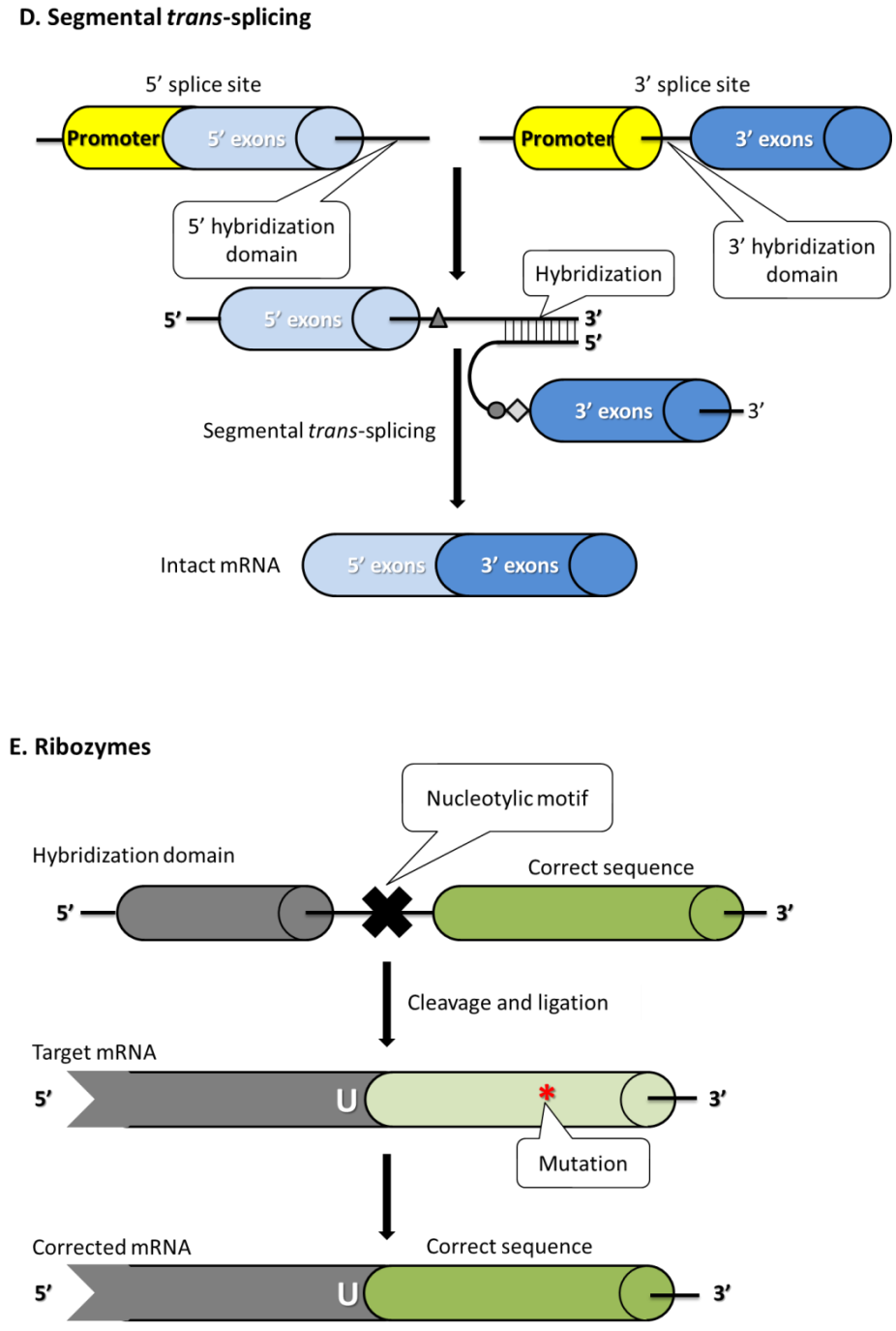


Fig. 18. RNA-modification strategies for genetic disorders.

4.3. Looking ahead: gene-inspired drug design and multimodal therapies

Researchers' ever-increasing knowledge of human genome, particular genes, and their disease-associated mutations has inspired various approaches to disease treatment design and discovery. Still a lot of scientific work is going on to find out the effective therapies for curing genetic diseases. The current treatments are not necessarily mutually exclusive, although in the meaning of some genetic investigators successful gene therapy would render other therapies redundant. These researchers are very enthusiastic about gene therapy, which has shown promising effects in clinical test. It is hoped to cure or improve treatment of genetic disorders by replacing the malfunctioned or mutated gene, manipulating or turning off the gene causing the disease or stimulate other physical functions to fight the disease. However, due to the above-mentioned potential side effects and inadequate proof for efficacy of gene therapy it may take some time before it will become available to the wider population of patients. On the other hand, by understanding the underlying molecular mechanisms linked to disorder, and also due to the fact that gene-based therapeutics continues to evolve, multimodal approaches to human genetic diseases are very likely scenario. Irrespectively, it is obvious that the future of genetic medicine will require close collaboration and multidisciplinary approaches, which certainly will be accompanied by unexpected interesting discoveries. Continuous progress in all these areas will certainly lead to the cure, rather than the care, of many genetic diseases.

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