Charles University

Faculty of Science

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Anastasiia Lushpina

Gene therapy in regenerative medicine
Genová terapie v regenerativní medicíně

Bachelor´s thesis

Supervisor: Mgr. Lydia Knight

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Prohlášení:

Prohlašuji, že jsem závěrečnou práci zpracovala samostatně a že jsem uvedla všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

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Podpis

Abstract

Gene therapy in regenerative medicine offers promising solutions for repairing or replacing damaged tissues and organs. This thesis describes the potential of gene therapy to revolutionize regenerative medicine by enabling targeted interventions at the molecular and genetic levels. The thesis delves into various gene therapy techniques such as viral and non-viral vectors, CRISPR-Cas9 gene editing, and RNA interference highlighting their application in regenerating tissues like bone, cardiac muscle, liver and nervous tissue. Through a review of current research, the thesis analyzes the efficacy, safety, and ethical considerations associated with these therapies, noting key breakthroughs and limitations that impact their translation to clinical practice.

Keywords: viral vector, non-viral vector, CRISPR/Cas9, regenerative medicine, genetic material, gene therapy, stem cell

Abstrakt v češtině

Genová terapie v regenerativní medicíně nabízí slibná řešení pro opravu nebo náhradu poškozených tkání a orgánů. Tato práce popisuje potenciál genové terapie pro revoluci v regenerativní medicíně tím, že umožňuje cílené intervence na molekulární a genetické úrovni. Práce se zabývá různými technikami genové terapie jako jsou virové a nevirové vektory, editace genu CRISPR-Cas9 a interference RNA a zdůrazňuje jejich použití v regenerujících tkáních, jako jsou kosti, srdeční sval, játra a nervová tkáň. Prostřednictvím přehledu současného výzkumu práce analyzuje účinnost, bezpečnost a etické úvahy spojené s těmito terapiemi, přičemž si všímá klíčových průlomů a omezení, která ovlivňují jejich převedení do klinické praxe.

Klíčová slova: virový vektor, nevirový vektor, CRISPR/Cas9 regenerativní medicína, genetický materiál, genová terapie, kmenová buňka

Contents

1 Introduction

Gene therapy in regenerative medicine represents a groundbreaking convergence of two fields: genetic modification and tissue regeneration. It involves using genetic material to repair, replace, or enhance the body's natural healing processes, aiming to restore function to damaged or diseased tissues and organs. Unlike traditional regenerative approaches, which often rely on stem cells, tissue engineering, or transplantation, gene therapy directly modifies the genetic makeup of cells to promote regeneration or prevent degeneration. At its core, gene therapy in regenerative medicine leverages advanced tools, like viral and non-viral vectors, gene-editing technologies such as CRISPR-Cas9, and cell-based therapies to either correct defective genes or introduce new ones that stimulate the body's regenerative capabilities. By reprogramming cells at a molecular level, gene therapy can enhance the repair and regeneration of tissues that have been damaged by injury, disease, or age-related degeneration. This approach is particularly promising in conditions where the body's natural regenerative ability is insufficient or impaired, such as in genetic disorders (e.g., muscular dystrophy, sickle cell anemia), degenerative diseases (e.g., osteoarthritis, heart disease), and even certain neurological conditions (e.g., spinal cord injuries, Parkinson's disease). Gene therapy can either directly repair the faulty genes that cause these conditions or deliver genes that encourage tissue regeneration, cell proliferation, and the restoration of normal function. By merging gene editing with the principles of regenerative medicine, this emerging field holds the potential to revolutionize how we treat not only genetic diseases but also age-related tissue damage, offering patients the possibility of cures and lasting solutions where previous therapies fell short.

2 Fundamentals of Regenerative Medicine and Gene Therapy

Regeneration refers to the biological process through which organisms restore or replace damaged or lost tissues, organs, or cells to regain normal function. It is a fundamental aspect of biology seen in various forms across species, from simple wound healing in humans to the remarkable ability of certain animals, like salamanders, to regrow entire limbs. Regeneration operates at the cellular and molecular levels, involving cell proliferation, differentiation, and tissue remodeling, guided by intrinsic genetic programs and external environmental signals. In humans, regenerative capacity is more limited. While tissues like the liver have significant regenerative potential, other organs, such as the heart and CNS, exhibit minimal capacity for natural regeneration. This limitation has driven the field of regenerative medicine, which seeks to harness biological processes and modern technologies to repair or replace damaged tissues and organs.

Gene therapy is an advanced medical approach that aims to treat or prevent disease by modifying human genetic material. It involves delivering, altering, or silencing specific genes in cells to correct genetic disorders, compensate for defective genes, or provide therapeutic benefits. Gene therapy methods include the use of viral vectors, non-viral vectors, and advanced gene editing tools such as CRISPR-Cas9. The applications of gene therapy are wide-ranging and include, for example, the treatment of inherited genetic disorders by correcting mutations that cause diseases such as cystic fibrosis (Crystal et al. 1994), sickle cell anemia (Pawliuk et al. 2001), or muscular dystrophy (Mendell et al. 2024), the treatment of cancer by modifying immune cells such as T cells to specifically target cancer cells (Tran et al. 2014), and the development of gene therapies to combat viruses such as HIV (Piché 1999; Rossi, June, and Kohn 2007). As gene therapy advances, it is increasingly being applied in broader contexts, including regenerative medicine, where it holds great promise for enhancing or enabling the body's regenerative capabilities. Gene therapy has become a transformative tool in regenerative medicine. While traditional regenerative approaches often rely on the transplantation of cells, scaffolds or biomaterials, gene therapy offers a molecular pathway to directly influence the regenerative process by stimulating tissue repair, namely introducing genes that promote cell proliferation, survival and differentiation to improve natural regeneration, correcting mutations that hinder regenerative abilities or cause degenerative conditions. In addition, it is also possible to engineer cells for therapeutic purposes. Reprogramming stem cells or somatic cells into desired cell types for tissue repair or organ regeneration. For example, in conditions such as Duchenne muscular dystrophy (Mendell et al. 2024), where muscle regeneration is impaired due to genetic mutations, gene therapy has been used to restore functionality by correcting faulty genes. Similarly, CRISPR technology has been explored to improve regenerative outcomes in damaged tissues such as the heart (Lebek et al. 2023) or bones (Arandjelovic et al. 2021), where intrinsic repair mechanisms are weak.

3 Stem Cells in Regeneration

Tissue regeneration is the process by which organisms repair and restore damaged or lost tissues, organs, or cells. It involves complex biological mechanisms at the cellular, molecular, and tissue levels. While the regenerative potential varies among different tissues and species, key mechanisms underlying regeneration include cell proliferation, differentiation, migration, and remodeling. These processes are regulated by intricate networks of signals, growth factors, and genetic programs. In response to injury, certain stem cells may become activated to repair damaged tissues. Stem cells have the unique ability to respond to injury and reprogram themselves to regenerate tissues, either by self-renewing or differentiating into functional cell types. By combining stem cell technology with gene therapy, a wide range of diseases and tissue regeneration needs can be addressed.

3.1 Embryonic Stem Cells (ESCs)

Human **embryonic stem cells**, derived from the inner cell mass of the blastocyst, have remarkable properties including pluripotency and self-renewal. These cells are able to proliferate *ex vivo* and at the same time retain the ability to differentiate into cells of all three germ layers: endoderm, mesoderm and ectoderm (Thomson et al. 1998; Itskovitz-Eldor et al. 2000). Using the differentiation properties of embryonic stem cells, a number of studies have been carried out to demonstrate the possibility of using ESCs for regeneration. For example, mouse ESCs have been engineered to create cells that produce insulin and other pancreatic endocrine hormones. The newly differentiated cells were able to form three-dimensional clusters similar to pancreatic islets. These clusters responded to glucose stimulation by releasing insulin, mimicking *in vivo* mechanisms (Lumelsky et al. 2001). In addition, it was experimentally proven that after transplantation of mouse ESCs into the rat spinal cord after injury (McDonald et al. 1999), the cells are able to survive and have the ability to differentiation into astrocytes, oligodendrocytes and neurons. The new neurons not only survived but were able to migrate up to 8 mm from the site of injury within 2-5 weeks. The pluripotent properties of embryonic stem cells are also confirmed by the formation of teratomas and benign tumors (Martin 1981). Despite the great potential of stem cells, their use is limited for ethical reasons. The main issue is the extraction of embryonic stem cells, which typically involves the destruction of a human embryo, which some individuals and groups equate to the taking of a potential human life. At the core of the debate is whether blastocyst-stage embryos have the moral status of humans. Additionally, many religious traditions view life as beginning at conception, making the destruction of embryos morally unacceptable. Some argue that research should focus on alternatives, such as iPSC, that do not involve embryos to avoid ethical conflicts.

3.2 Adult Stem Cells

Hematopoietic stem cells (HSCs) are a type of stem cell that gives rise to all other blood cells during the process of hematopoiesis. They are found in bone marrow (Till and McCulloch 1961) or liver (Taniguchi et al. 1996). An important property of hematopoietic stem cells is self-renewal. However, when doing so, it is important to maintain a state of rest and avoid dividing too frequently. To maintain a quiescent state, HSCs use the cell cycle inhibitor p21. In the absence of p21, the cycle is intensified, which in turn leads to cell

exhaustion and death (Cheng et al. 2000). An equally important role is played by the cells surrounding the HSCs. For example, megakaryocytes maintain HSC quiescence during homeostasis and promote regeneration and prevent HSC death following chemotherapeutic stress (Zhao et al. 2014). These adult bone marrow cells have enormous differentiating capacity because they can also differentiate into epithelial cells of the liver, lung, gastrointestinal tract, and skin (Krause et al. 2001). For example, a study showed that intravenous injection of adult bone marrow cells into FAH(-/-) mice, an animal model of type I tyrosinemia, saved the mouse and restored the biochemical function of its liver (Lagasse et al. 2000). As with other allogeneic transplantations, the main problems of HSC transplantation are the low rate of donor engraftment and the high risk of graft-versus-host disease (GVHD). Transplantation of purified allogeneic HSCs reduces the risk of GVHD compared to unpurified ones, since it does not contain mature elements (Shizuru et al. 1996) but results in delayed engraftment (Zheng et al. 2011). While quiescence is vital for HSC health and function, gene therapy inherently poses risks by disrupting this state. To enhance gene editing efficiency, strategies may be employed to activate HSCs from quiescence, encouraging them to divide. However, forcing HSCs to divide can carry the risk of damaging their self-renewal capacity or even triggering transformation into cancerous cells (C. Chen et al. 2008; Mortensen et al. 2011; Mantel et al. 2015; Ito et al. 2016; Ho et al. 2017; Verovskaya, Dellorusso, and Passegué 2019). Overactivation can potentially lead to the depletion of the stem cell pool, reducing long-term engraftment and blood production. Balancing therapeutic efficacy with the preservation of HSC quiescence and genomic stability is crucial for the safe and effective application of HSC-based gene therapies.

Mesenchymal stem cells (MSCs) are a type of multipotent stem cells that can differentiate into various cell types, including osteoblasts, chondrocytes, cardiomyocytes and adipocytes. MSCs are found in several tissues, including bone marrow (Dennis et al. 2002), menstrual fluid (Hida et al. 2008), adipose tissue (Zuk et al. 2002), umbilical cord blood, and placenta. Differentiation is regulated by genetic events involving different proteins such as vascular endothelial growth factor (VEGF) (Asahara et al. 1999), HMG-CoA reductase inhibitor (Llevadot et al. 2001) and SDF-1 (Unzek et al. 2007), and specific regulatory genes can induce progenitor cells to differentiate into a particular lineage. Also a significant number of MSCs can be found in the hematopoietic stem cell niche in the bone marrow, where they stimulate the HSCs and support hematopoiesis (Méndez-Ferrer et al. 2010). MSCs demonstrate immunomodulatory properties. These include inhibition of T cell, B cell, and natural killer cell proliferation and function. This nonspecific suppression is achieved primarily by inhibition of cyclin D2 (Siegel, Schäfer, and Dazzi 2009). Co-infusion of MSCs with allosplenocytes prolonged the mean survival time of healthy recipient mice reduced histopathology associated with graft-versus-host disease compared with controls without MSCs (H. Li et al. 2007).

Neural stem cells (NSCs) are a type of multipotent stem cells found in the central (CNS) and peripheral nervous system (PNS). They have the ability to differentiate into three main types of CNS cells: neurons, astrocytes and oligodendrocytes. The human hippocampus retains the ability to generate neurons throughout life (Eriksson et al. 1998; Spalding et al. 2013). This phenomenon is known as adult hippocampal neurogenesis

and plays a key role in learning and memory. To differentiate NSCs, they use various transcription factors and enzymes, such as DNA methyltransferase 1 (DNMT1) (Noguchi et al. 2015), Sox2 (Ferri et al. 2004), MeCP2 (Smrt et al. 2007). Separately, we can highlight Notch, whose signaling can be divided into two stages. Notch initially inhibits neuronal fate while promoting glial cell fate. In the second step, Notch promotes astrocyte differentiation while inhibiting the differentiation of both neurons and oligodendrocytes (Grandbarbe et al. 2003). Neural stem cell-based gene therapy holds great promise for regenerating the nervous system in conditions like spinal cord injuries (Salazar et al. 2010; Nemati et al. 2014), neurodegenerative diseases (L. Xu et al. 2006; W. Zhang et al. 2015), and brain trauma (Duan et al. 2016; Portnow et al. 2017). However, there are significant limitations associated with the use of NSCs, such as limited access to source material, high costs for cultivation, and challenges related to cell number and integration. While these limitations present obstacles to widespread clinical application, ongoing research into stem cell biology, gene editing technologies, and supportive therapies may improve the feasibility of NSC-based regenerative treatments in the future. Alternative approaches like iPSCs, CRISPR technology, and combination therapies with biomaterials offer exciting avenues for overcoming some of these challenges.

4 Gene Therapy Techniques in Regenerative Medicine

Gene therapy is a groundbreaking approach in regenerative medicine that involves delivering genetic material into cells to restore, modify, or enhance cellular functions. This approach holds tremendous promises for treating genetic disorders, promoting tissue regeneration, and enabling precise control over cellular behavior to repair damaged tissues or organs.

4.1 Gene Delivery Methods

Gene delivery refers to the process of introducing genetic material (DNA or RNA) into cells to correct or modify genetic defects, enhance cellular function, or facilitate research. This process is essential in gene therapy, genetic research, and biotechnology applications. There are several methods of gene delivery, broadly categorized into viral and non-viral systems. Each method has its unique advantages, limitations, and applications. Typically, we need a delivery vector for nucleic acid delivery. This is because the passage of DNA/RNA directly through the plasma membrane is difficult due to the size and charge of these molecules. The vectors are tools designed to carry genetic material into cells effectively. These vectors can be broadly categorized into viral vectors and non-viral vectors, each tailored for specific applications in gene therapy, research, and biotechnology. Below are the most frequently used vectors (Fig. 1).

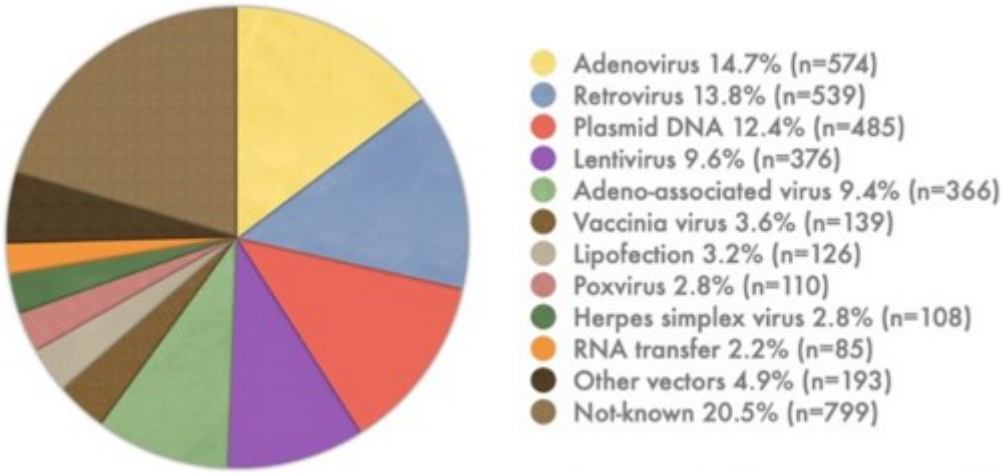

Figure 1. Information about 3900 protocols of gene therapy over the world in 2023. Adenovirus and retrovirus are the most commonly used viral vectors in gene therapy. Reprinted from Gene Therapy Clinical Trial Worldwide (Journal of Gene Medicine) http://www.abedia.com/wiley.

4.1.1 Viral Vectors

If we perceive a virus as genetic information in a protein coat, then the viral vector will be this protein coat, devoid of a large part of the viral genome. The virus for the viral vector must be selected individually for each case. Different viruses are selected based on their effectiveness in targeting certain types of cells and their ability to carry a large genetic load. Commonly used vectors include adenoviruses (AdVs), adeno-associated viruses (AAVs) and retroviruses (Fig. 1), (Tab. 1).

Table 1. Comparison of commonly used viral vectors

Viral Vector Type	Adenovirus (AdV)	Adeno-associated virus (AAV)	Retrovirus (lentivirus)
Genome	dsDNA (Green et al. 1967)	ssDNA (Berns and Rose 1970)	ssRNA (Panganiban and Fiore 1988)
Packing capacity	Up to 8–36 kb	Up to 4.7 kb	Up to 8 kb (Vogt and Simon 1999)
Target Cells	Broad natural tropism for epithelial, ocular and immune cells	Liver, muscle, neurons (Zinn et al. 2015; Santiago-Ortiz et al. 2015)	Only non-dividing cells
Integrate with host genome	No	Yes (Kotin et al. 1991)	Yes (Engelman, Mizuuchi, and Craigie 1991)
Risk of Insertional Mutagenesis	Low	Low	Hight (Carr and Campbell 1958; Franz et al. 1985)
Duration of Expression	Short-term (need helper) (Ehrhardt and Kay 2002)	Long-term (M. G. Kaplitt et al. 1994), (Michael G Kaplitt et al. 1996; Svensson et al. 1999)	Long-term (genome integration)
Immunogenicity	Hight (Zsengellér et al. 2000)	Low (Chirmule et al. 1999)	Moderate (Brown et al. 2007)

After selecting a suitable virus, the viral particle must be prepared for further gene transfer. Preparation and integration include several phases (Kimura et al. 2019). First, it is necessary to insert the therapeutic gene into the viral genome. Nonessential viral genes are often deleted to make room for the therapeutic gene and prevent the virus from replicating, which in turn will reduce the likelihood of the immune system reacting to the virus. The modified viral genome is packaged into viral particles. This involves the production of viral particles in the cell line that can support viral replication and packaging. Viral particles are introduced into target cells either *ex vivo* or *in vivo*. After the virus binds to specific receptors on the surface of target cells and is taken up by endocytosis or direct fusion with the cell membrane. In the case of retroviruses and lentiviruses, the viral genome is integrated into the genome of the host cell, providing stable and long-term expression of the therapeutic gene. In the case of adenoviruses and AAVs, viral DNA exists as an episome (a single DNA molecule within the nucleus) and is not integrated into the host genome, resulting in transient expression. Once inside the cell, the therapeutic gene is transcribed and translated into the desired protein, correcting a genetic defect or providing therapeutic benefits. Viral vectors remain indispensable in modern gene therapy and research due to their high efficiency and versatility. Choosing the right vector depends on the target cells, therapeutic goals, and safety considerations. Advances in engineering safer and more efficient viral vectors continue to push the boundaries of their applications.

4.1.2 Non-Viral Vectors

Non-viral vectors offer a promising alternative to viral vectors for the delivery of genetic material into cells. These vectors utilize physical, chemical, or biological methods to introduce therapeutic genes or gene-editing tools into target cells. They are increasingly favored for their reduced immunogenicity, scalability, and safety in gene therapy applications.

Chemical non-viral vectors, which rely on synthetic or natural materials, deliver genetic material (DNA, RNA, or CRISPR components) into cells through complex formation or carriers. These carriers protect genetic material from degradation, enhance cellular uptake, and release the payload inside the target cells. Liposomes and lipid nanoparticles (LNPs) are two key non-viral vectors in gene therapy. Liposomes, spherical vesicles composed of lipid bilayers, are versatile and can carry a wide range of genetic materials (Bangham, Standish, and Watkins 1965). LNPs, on the other hand, are more advanced lipid-based carriers that offer higher efficiency in encapsulating and delivering nucleic acids. Though LNPs show great promise, they can still cause toxicity and immune responses, particularly with repeated use (Filion and Phillips 1997; Kedmi, Ben-Arie, and Peer 2010). Both liposomes and LNPs present valuable options, with LNPs currently taking a leading role in mRNA-based therapies, particularly in vaccines (Kremsner et al. 2022; Rowe et al. 2023). Inorganic nanoparticles (NPs) are another emerging class of non-viral vectors. These engineered nanoparticles offer high stability, ease of surface modification, and multifunctionality. Gold nanoparticles (AuNPs), for example, are biocompatible and can be functionalized to deliver nucleic acids (T. Zhang et al. 2011). They offer enhanced stability and cellular uptake but may face challenges related to aggregation and bioaccumulation (Khlebtsov and Dykman 2011). Inorganic nanoparticles hold considerable potential in gene therapy, with ongoing research aimed at improving their biocompatibility, targeting efficiency, and scalability for clinical use.

Physical non-viral vectors rely on physical methods to deliver genetic material. Techniques like electroporation, gene guns (biolistics), and direct injection of naked DNA/RNA create temporary disruptions in the cell membrane or mechanically introduce genetic material directly into target cells. Electroporation, which uses electrical pulses to create transient pores in cell membranes, has become popular for its high efficiency in delivering nucleic acids into various cell types (Neumann et al. 1982; Titomirov, Sukharev, and Kistanova 1991). Gene guns (Yang et al. 1990; Klein et al. 1992)and direct injection methods (Wolff et al. 1990) are simpler approaches, though they face limitations in delivery efficiency and targeting precision (Sudha et al. 2001; Goudy, Wang, and Tisch 2008). While physical methods avoid the immunogenic risks of viral vectors, challenges related to targeting, scalability, and cell viability remain (P.-W. Lee et al. 2008).

These methods offer advantages in safety, scalability, and reduced immune responses, though each comes with its own set of challenges, such as efficiency, stability, and toxicity. Continued research and optimization are crucial to improving the effectiveness and applicability of these non-viral delivery systems in treating genetic diseases, cancers, and other conditions. As these methods evolve, they hold significant promise for advancing gene therapy technologies.

4.2 Gene Editing Techniques

Gene editing is a set of technologies that allow scientists to make precise modifications to an organism's DNA. These modifications can involve adding, removing, or altering specific sections of the DNA sequence. DNA editing has significant potential in fields such as medicine, agriculture, and basic research, allowing for new approaches to treating genetic and acquired diseases, as well as better understanding the function of genes. DNA editing typically involves making precise cuts in the DNA at a specific location. Once the DNA is cut, the cell's natural repair processes are triggered. There are two primary pathways cells use to repair double-strand breaks (DSBs) in DNA. The first is Non-Homologous End Joining (NHEJ). This is a quick but error-prone repair process that often introduces small insertions or deletions, potentially "knocking out" a gene (Boulton and Jackson 1996; Budman and Chu 2005). The second is Homology-Directed Repair (HDR). This repair process is more accurate but only occurs in certain cell types and conditions, as it requires a homologous DNA region as a template for repair. The template is usually a sister chromatid, which is only available in dividing cells. Also HDR can use a provided DNA template to insert or "knock in" new genetic material precisely at the target location (Saleh-Gohari and Helleday 2004; F. Chen et al. 2011). We can harness these repair processes to make specific changes to the DNA (Fig. 2A). Several tools have been developed to facilitate precise DNA editing. Below we will look at a few of them and compare them with each other.

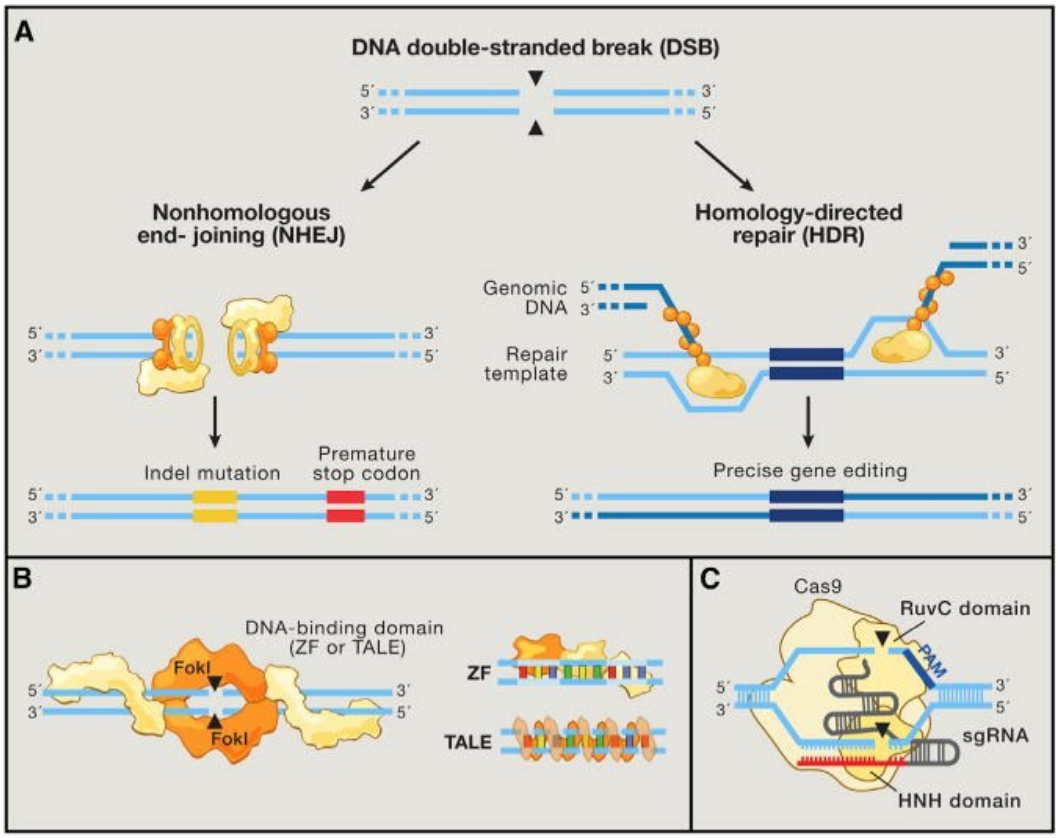

Figure 2. Genome Editing Technologies Exploit Endogenous DNA Repair Machinery. (A) Natural pathways for repairing double-strand DNA breaks (DSBs); (B) Editing by ZFNs (Zinc Finger Nucleases) and TALENs (Transcription Activator-Like Effector Nucleases); (C) CRISPR – Cas9 DNA editing. Guide RNA (red) is localized to specific DNA sequences, directing the endonuclease Cas9 to its target. Direct cleavage of the DNA chain occurs on specific protospacer adjacent matif (PAM, blue). (Hsu, Lander, and Zhang 2014).

4.2.1 ZFNs (Zinc Finger Nucleases)

Zinc Finger Nucleases (ZFNs) are a class of engineered DNA-binding proteins used for targeted genome editing. ZFNs combine the DNA-binding ability of zinc finger proteins with the nuclease activity of the FokI enzyme to induce DSBs at specific sites in the genome. These breaks can then be repaired by cellular repair mechanisms, such as NHEJ or HDR, which allows for gene knockout, insertion, or correction. Zinc finger proteins are naturally occurring DNA-binding proteins that recognize and bind to specific DNA sequences (Miller, McLachlan, and Klug 1985). This structurally simple ββα domain is stabilized by hydrophobic interactions and the coordination of a zinc ion by the eponymous cysteine and histidine residues. Each zinc finger domain typically recognizes a 3-base pair sequence of DNA, and multiple zinc fingers are used in a ZFN to target longer DNA sequences with greater precision (Pavletich and Pabo 1991). The number of zinc finger domains determines the target sequence length. Usually, 6 zinc fingers are used to target an18 base pair sequence. This number of nucleotides is sufficient to find any unique locus in any known genome (Liu et al. 1997). The FokI nuclease is an enzyme that cleaves DNA, but it requires the dimerization of two FokI domains to function. In ZFNs, the FokI domain is linked to the zinc finger DNA-binding domain. When the zinc fingers bind to the target DNA sequence, the two FokI domains come together and create a DSB in the DNA (Kim, Cha, and Chandrasegaran 1996) (Fig. 2B). Although ZFNs were among the first genome-editing technologies, they have been largely superseded by TALENs and CRISPR-Cas9 due to their simplicity and lower cost. However, ZFNs remain valuable in specific applications, such as therapeutic gene editing, where high precision and the absence of PAM dependency are critical.

4.2.2 TALENs (Transcription Activator-Like Effector Nucleases)

TALENs (Transcription Activator-Like Effector Nucleases) are a genome-editing tool that uses engineered proteins to bind and cleave specific DNA sequences. TALENs are composed of two key components: TAL effector proteins, which bind to DNA, and the FokI nuclease, which creates a DSB in the DNA at the target site. These breaks can be repaired by the cell's natural mechanisms, enabling the editing of the genome. TALEs are proteins originally derived from Xanthomonas bacteria, where they help the pathogen infect plant cells by binding to specific DNA sequences (Szurek et al. 2002). This domain recognizes DNA sequences through repeat-variable diresidues (RVDs). Each RVD corresponds to a single nucleotide in the DNA sequence: NI recognizes adenine (A), HD recognizes cytosine (C), NG recognizes thymine (T), NN recognizes guanine (G) or adenine (A) (Moscou and Bogdanove 2009). A series of these modular repeats can be customized to target a specific DNA sequence. TALENs use the FokI nuclease, which requires dimerization (pairing of two TALENs) to cut DNA. This ensures that DNA cleavage occurs only when two TALENs bind at adjacent sites on opposite DNA strands, enhancing specificity (Fig. 2B). TALENs are powerful and precise genome-editing tools, offering high specificity and flexibility for targeting diverse DNA sequences. Despite challenges like design complexity and delivery limitations, they remain a valuable alternative to CRISPR-Cas9, particularly in applications requiring precise, PAM-independent targeting. Their use in therapeutic and agricultural contexts continues to grow as delivery methods and design processes improve.

4.2.3 CRISPR-Cas9

CRISPR-Cas9, which stands for Clustered Regularly Interspaced Short Palindromic Repeats and CRISPR-associated protein 9, was initially discovered in bacteria and archaea. Prokaryotes use CRISPR sequences as a form of immune memory, storing fragments of DNA (spacer) from viruses that have attacked them. When the same virus invades again, the prokaryote uses these stored sequences to create RNA guides, which direct a Cas protein to cut the viral DNA, effectively disabling the virus (Bolotin et al. 2005; Barrangou et al. 2007). In the natural CRISPR-Cas9 system, there are two RNA molecules acting as gRNA (guide RNA). CrRNA (CRISPR RNA), which is complementary to targeted DNA and tracrRNA (trans-activating CRISPR RNA), which is necessary for stabilization and activation of the RNA-protein complex (Deltcheva et al. 2011). In addition to two RNA molecules, it is also possible to use chimeric, single-stranded RNA to deliver the cas9 endonuclease. It programs cas9 more effectively and is easier to produce. Direct binding to DNA is provided by the 5'-terminal 20 nucleotides of the gRNA (Jinek et al. 2012). The researchers were able to adapt this system to target and edit genes in eukaryotic cells, making it a powerful tool in biotechnology and medicine. Simply put, CRISPR-Cas9 functions as a molecular toolkit to edit specific DNA sequences. The guide RNA in this case is a synthetic RNA sequence that is designed to match the target DNA sequence. The gRNA guides the Cas9 enzyme to the exact location in the genome. Cas9, as in the case of its natural role, cuts both strands of DNA at the target site and creates a DSB (Garneau et al. 2010; Jinek et al. 2012; Cong et al. 2013). After localization of the complex to DNA, the RuvC (DNA (+) strand cleavage) and HNH domains DNA (-) strand cleavage (Gasiunas et al. 2012) of the Cas protein cleave the DNA strand at the PAM (Protospacer Adjacent Motif). The PAM is a specific sequence of nucleotides (usually about 3) located immediately adjacent to the target DNA sequence. The Cas9 protein requires the presence of the PAM sequence to bind to DNA and initiate the cleavage process. Cas9 will not bind to or cut a DNA sequence if the PAM sequence is not present near the target site.(Mojica et al. 2009) (Fig. 3C). CRISPR-Cas9 is a transformative tool in modern science, enabling precise genome editing. Despite challenges like off-target effects and ethical considerations, ongoing advancements promise to unlock its full potential, shaping the future of genetic research and therapy. CRISPR-Cas9 stands out as the most transformative DNA editing technology, driving advancements in research, medicine, and biotechnology (Tab. 2).

Table 2. Comparison of different DNA editing methods

	ZFN	TALEN	CRISPR-Cas9
Mechanism	Combines zinc finger proteins (DNA-binding domains) with FokI nuclease to target DNA.	Uses TAL (Transcription Activator-Like) effector proteins fused with FokI nuclease to target DNA.	Uses guide RNA (gRNA) to direct the Cas9 nuclease to a specific DNA sequence.
Target Range	Broad; not limited by PAM but depends on the	Broad; no PAM requirement, offering	Limited by the need for a PAM sequence (e.g., NGG for SpCas9).

	availability of appropriate zinc fingers.	greater flexibility than CRISPR.	
Length of target site	18 nt (Liu et al. 1997).	35 nt (Cermak et al. 2011)	20 nt (Jinek et al. 2012)
Specificity	Moderate; influenced by zinc finger accuracy.	Very high; highly specific protein-DNA interactions due to modular TAL repeats.	High, determined by the gRNA and PAM sequence; risk of off-target effects.
Ease of Design	Complex; requires engineering zinc finger proteins specific to each target sequence.	Moderate; designing TAL repeats is less complex than ZFNs but still requires protein engineering	Simple; requires design of synthetic guide RNA and Cas9 nuclease.
Advantages	- Earlier tool with proven applications. - No PAM dependency.	- Highly specific. - Can target virtually any sequence. - Suitable for complex genomes.	- Simplicity and ease of use. - Rapid advancements in tools and variants. - Cost-effective.
Limitations	- Difficult and expensive to design. - Limited to fewer labs with expertise.	- More expensive and time-consuming than CRISPR. - Protein design challenges for each target.	- Requires PAM sequence. - Off-target risks. - Relies on repair mechanisms for precision.

4.3 RNA Interference and Gene Silencing Techniques

RNA interference and gene silencing techniques are innovative approaches in regenerative medicine, offering precise control over gene expression. By selectively silencing harmful or undesired genes, these technologies can modulate cellular behavior, enhance tissue repair, and address diseases associated with abnormal gene activity.

4.3.1 RNA Interference

RNA interference (RNAi) is a biological process in which RNA molecules inhibit gene expression by neutralizing targeted messenger RNA (mRNA) molecules (Izant and Weintraub 1984). This process is a vital mechanism for regulating gene expression (A. Fire et al. 1991) and defending against viruses in eukaryotic cells (Ratcliff, Harrison, and Baulcombe 1997). There are two key components to gene silencing. The first is non-protein-coding RNA. siRNA (small interfering RNA) and miRNA (microRNA) are both short RNA molecules involved in regulating gene expression through the RNAi pathway. SiRNAs are exogenous molecules , typically 21 – 23 base pairs in length, that are introduced into cells artificially or derived from

viral infections (Zamore et al. 2000; Elbashir, Lendeckel, and Tuschl 2001). It is double-stranded, with perfect sequence complementarity to their target mRNA on the guide strand and the identity of the target mRNA on the other, passenger strand (Andrew Fire et al. 1998). This high specificity enables siRNA to silence a single gene. In contrast, miRNAs are endogenously encoded by the genome and naturally processed, however, it is single-stranded and exhibit partial complementarity to their targets, allowing them to regulate multiple genes simultaneously. Once introduced into cells or, as in the case of miRNA, transcribed within the cell, the RNA is sliced into smaller pieces. siRNAs are typically processed from longer double-stranded RNA by the enzyme Dicer, while miRNAs are transcribed as primary miRNA (pri-miRNA) in the nucleus and sequentially processed by Drosha (Y. Lee et al. 2003) and Dicer (Bernstein et al. 2001). The second essential component in RNA interference is the Argonaute proteins. They are an essential component of the RNA-induced silencing complex (RISC). Argonaute proteins bind small RNA molecules and form a stable guide strand complex. Then the guide RNA directs the Argonaute protein to complementary mRNA sequences through Watson-Crick base-pairing. Gene silencing can be achieved through endonuclease activity, namely Argonaute proteins with slicer activity (e.g., AGO2) cleave the target mRNA, leading to its degradation (Hammond et al. 2000) or, in the miRNA pathway, Argonaute proteins repress translation or promote mRNA decay without slicing. The simplicity of the mechanism based on Watson-Crick pairing makes this method highly specific, and the simplicity of the siRNA/miRNA design allows customization for virtually any gene. The simplicity of the design does not negate off-target effects (Jackson et al. 2003), so poorly designed siRNA or miRNA can bind non-specifically to unintended mRNA targets. Other challenges faced by this method include choosing an effective delivery method (Semple et al. 2010) and immune response (Karikó et al. 2004), exogenous siRNA can trigger unwanted immune activation in some cases. In addition, siRNA is susceptible to degradation by nucleases in biological systems (Song et al. 2003). RNAi has revolutionized gene-silencing research, such as siRNA aimed at p53 to prevent kidney injury (Molitoris et al. 2009) and caspase-2, where siRNA acts as a neuroprotector and prevents the loss of retinal ganglion cells after damage to the optic nerve (Ahmed et al. 2011). It can also be used for vascular regeneration (Zhou et al. 2016) or prevention of coronary heart disease (Tadin-Strapps et al. 2015) RNAi holds great promise for therapeutics, but challenges in delivery and specificity remain areas of active investigation.

4.3.2 Antisense Oligonucleotides (ASOs)

Antisense oligonucleotides (ASOs) are short, synthetic strands of nucleic acids designed to selectively bind to specific RNA molecules via complementary base pairing (Stephenson and Zamecnik 1978). By binding to their target RNA, ASOs modulate gene expression at the RNA level, offering a promising approach for treating various genetic and acquired diseases. ASOs can influence RNA function in several ways. Given that ASOs can function both in the cytoplasm and in the nucleus, ASOs target both mRNA and pre-mRNA. The appropriate mechanism of action on the transcript is selected depending on the desired result. For example, inside the nucleus, ASOs can alter pre-mRNA splicing by blocking splicing sites or regulatory sequences (Smith et al. 1986). This approach is useful for diseases caused by splicing defects, such as spinal muscular atrophy (SMA) (Passini et al. 2011). Similarly, ASOs can inhibit translation by binding to the start codon

(Talbot et al. 2010) or other regions of mRNA (Wheeler et al. 2009; Mulders et al. 2009; Lundblad and Altman 2010), ASOs can block ribosome assembly (Braasch, Liu, and Corey 2002), preventing protein synthesis. Finally, ASOs can reduce protein levels by targeted mRNA degradation, by recruiting RNase H binds (Agrawal et al. 1990) to target mRNA and induce its cleavage. RNase H specifically recognizes DNA-RNA hybrids (Stein and Hausen 1969) and degrades the RNA strand.

In summary, while both ASOs and RNAi aim to reduce or modulate gene expression, their mechanisms, molecular requirements, and applications differ significantly. ASOs offer flexibility in target location (nucleus and cytoplasm) and action (degradation or splicing modification), while RNAi is cytoplasmic and relies on RISC-induced mRNA degradation. Most ASOs do not naturally penetrate cell membranes easily, so they often require delivery systems (such as liposomes (S et al. 2019), nanoparticles (Jewell et al. 2011), or conjugation to cell-penetrating peptides (Abes et al. 2006; Shiraishi and Nielsen 2006)) to enhance uptake. ASOs are typically unstable in biological environments and are rapidly degraded by nucleases. To enhance their stability, chemical modifications (such as phosphorothioate backbones (Agrawal et al. 1990) or 2'-O-methyl modifications (Raal et al. 2010)) are often required. These modifications can improve their stability but may also affect the pharmacokinetics and toxicity profiles (Goemans et al. 2011) of ASOs. Although ASOs are designed to be highly specific, there is still the potential for off-target binding (Yoshida et al. 2019) or unintended interactions with other RNA molecules. This can lead to nonspecific silencing or unintended effects on cellular processes, such as alternative splicing (van Deutekom et al. 2007; Cirak et al. 2011) or other regulatory pathways (Hedaya et al. 2023). Careful design and validation are required to minimize these risks.

4.4 Cellular Reprogramming for Regeneration

Cellular reprogramming is a transformative approach in regenerative medicine that involves reprogramming somatic (adult) cells into pluripotent or lineage-specific cells. This strategy aims to generate cells capable of repairing or replacing damaged tissues, enabling novel treatments for degenerative diseases, injuries, and age-related disorders. Cellular reprogramming leverages advanced techniques in molecular biology to reverse or redirect cellular fate.

4.4.1 Induced Pluripotent Stem Cells (iPSCs)

Induced pluripotent stem cells (iPSCs) are a type of stem cell that can be obtained directly from adult cells. iPSCs are similar to ESCs in surface antigens, morphology, and gene expression. These cells are important because they have the ability to differentiate into a wide variety of cell types, similar to embryonic stem cells, but without the associated ethical issues. The process of creating iPSCs involves reprogramming adult cells to return to a pluripotent state. This is usually done by introducing certain genes or proteins that are critical for maintaining the basic properties of embryonic stem cells. The most common method involves the use of four transcription factors: Octamer-binding transcription factor 4 (OCT4), SRY (sex-determining region Y)-box 2 (SOX2), Kruppel-like factor 4 (KLF4), Myc proto- oncogene protein (KLF4). These factors can be introduced using various methods such as viral vectors, plasmid transfection or protein-based methods. iPSCs avoid ethical problems associated with the use of embryonic stem cells due to the fact that the source of cells

for iPSCs can be cells obtained from the patient's own cells, which also reduces the risk of immune rejection. It is important to responsibly select somatic cells for reprogramming; iPSCs are assumed to contain residual DNA methylation characteristics characteristic of their somatic cells. This promotes differentiation of iPSCs along lineages associated with the donor cell, while simultaneously limiting alternative developmental pathways. The table below shows examples of cells that can be used for reprogramming and the factors that cause it (Tab. 3). iPSCs have revolutionized stem cell research and opened new avenues for personalized medicine, disease modeling, and therapeutic development. Continued advancements in reprogramming techniques and understanding of iPSC biology are critical for overcoming current limitations and realizing their full potential, such as for the restoration of the retinal pigment epithelium (Leach et al. 2016) and the treatment of neurodegenerative diseases (Doi et al. 2020).

Table 3. Cell Origin of iPS Cells (L. Ye, Swingen, and Zhang 2013).

Cell Source	Vector	Reprogramming Factors
Human ADS (Shimada et al. 2012)	Retrovirus Nonviral minicircle	Mouse Oct4, Sox2, Klf4, and L-Myc Oct4, Sox2, Nanog and LIN28
Human CD34+ blood cells (Z. Ye et al. 2009)	Retrovirus	Oct3/4, Sox2, Klf4, and c-Myc
Human CD34+ cord blood cells (Takenaka et al. 2010)	Retrovirus	Oct4, Sox2, Klf4, and c-Myc
Human dental tissue cells (Yan et al. 2010)	Retrovirus	Oct4, Sox2, Klf4, c-Myc or Lin28,Nanog, Oct4, Sox2
Human skin fibroblast (Takahashi et al. 2007)	Retrovirus	human Oct3/4, Sox2, Klf4, and c-Myc
Human somatic cells(Yu et al. 2007)	Lentivirus	Oct4, Sox2, Nanog, and LIN28
HUVEC (Panopoulos et al. 2011)	Retrovirus	Oct3/4, Sox2, Klf4, and c-Myc
Human oral mucosa fibroblasts (Miyoshi et al. 2010)	Retrovirus	Oct3/4, Sox2, Klf4, and c-Myc

4.5 *Ex Vivo* vs. *In Vivo* Gene Therapy

Gene therapy approaches can be broadly categorized into *ex vivo* and *in vivo* methods based on how genetic material is delivered to the target cells. In *ex vivo* gene therapy, cells are removed from the patient, genetically modified outside the body and then reintroduced into the patient. In *in vivo* gene therapy, genetic material is directly delivered to the target cells within the patient's body (Fig. 3).

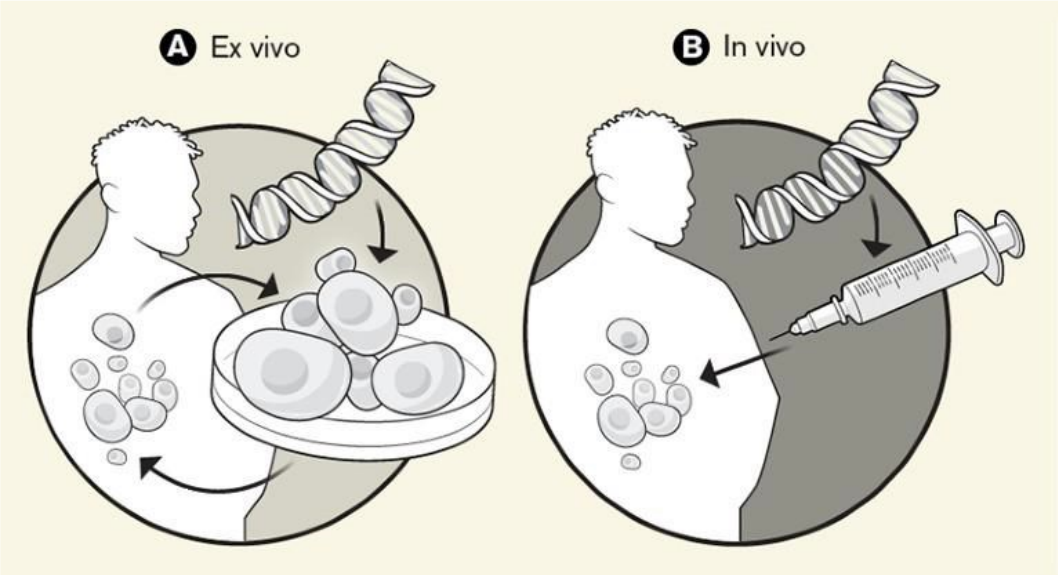

Figure 3. Location of gene therapy (Landhuis 2021).

Both *ex vivo* and *in vivo* gene therapies offer unique advantages and are suited for different applications. *Ex vivo* methods provide precise control and safety for certain cell types, while *in vivo* approaches are more practical for targeting internal tissues and organs. The choice depends on the disease, target cells, and required precision. Combining these approaches is a promising direction for advancing gene therapy (Tab.4).

Table 4. Comparison of *ex vivo* and *in vivo* gene therapy

Factor	*Ex Vivo* Gene Therapy	*In Vivo* Gene Therapy
Accessibility	Requires accessible cells (e.g., blood)	Suitable for inaccessible tissues/organs
Control	High precision and pre-validation	Limited control over delivery
Immune Response	Lower risk due to controlled reinfusion	Higher risk from direct vector exposure
Complexity	Requires specialized facilities	Easier to administer
Long-Term Expression	Possible with integrating vectors	Dependent on vector type
Disease Suitability	Blood, immune disorders, stem cell therapy	Organ-specific or systemic conditions
Scalability	Limited to personalized treatments	Highly scalable for broader application

5 Applications of Gene Therapy in Regenerative Medicine

Gene therapy in regenerative medicine aims to harness the body's own regenerative capabilities by correcting genetic defects, promoting cell survival and function, and enhancing repair and regeneration of tissues and organs. Although these approaches are still in their early stages, they have enormous potential to treat a wide range of debilitating conditions.

5.1 Bone regeneration

Bone tissue is specialized connective tissue that forms the structural framework of the bones of the vertebrate skeleton. It is a dynamic and complex tissue with several important functions, including providing support, protecting internal organs, facilitating movement, storing and releasing minerals, and housing bone marrow. Bone regeneration is a complex physiological process that involves replacing lost or damaged bone tissue. This process may occur naturally, such as during the healing of a fracture, or it may be facilitated by medical intervention. Bone regeneration is critical to maintaining the integrity and function of the skeletal system. Bone regeneration can be divided into four distinct phases (Einhorn 1998):

1. Inflammation. Immediately after a fracture, blood vessels in the bone and surrounding tissue rupture, causing a hematoma (blood clot) to form around the fracture site. Inflammatory cells enter the area to remove dead tissue and release cytokines and growth factors that initiate the healing process.

2. Formation of soft callus. Within a few days, the hematoma is replaced by granulation tissue, which gradually transforms into a soft callus consisting of collagen and fibrocartilage. This process is associated with mesenchymal stem cells throughout the callus (Brighton and Hunt 1991). This soft callus stabilizes the fracture but is not yet strong enough to support weight.

3. Formation of hard callus. Osteoblasts also emerge from mesenchymal stem cells (Brighton and Hunt 1991) which begin to produce new bone, forming a hard callus of interwoven bone. This stage lasts from several weeks to months, during which a hard callus closes the fracture gap, partially restoring the strength and stability of the bone.

4. Remodeling. Osteoblasts produce two molecules important for remodeling, namely RANK ligand (RANK-L) and its binding neutralizing soluble decoy receptor, osteoprotegerin (OPG). Receptor activator of NF-kappaB (RANK) is present on the surface of osteoclast precursor cells, where its interaction with RANK-L causes them to differentiate into osteoclasts, thereby increasing bone destruction. Woven bone is gradually replaced by lamellar bone, which is stronger and more organized. Osteoclasts resorb excess bone formed during the healing process, and osteoblasts lay down new bone in an orderly manner, restoring the original shape and structure of the bone. Osteoclast activation is induced by binding of RANK-L to OPG (Gori et al. 2000).

Gene therapy for bone regeneration usually involves delivering specific genes to cells at the site of a bone injury or defect. These genes code for proteins that promote bone growth and healing. The most commonly targeted genes are those that encode growth factors. For example, stromal cells infected with a retrovirus containing BMP4 completely reversed a critical calvarial defect in rats (Gysin et al. 2002). Also, bone marrow

stromal cells (BMSCs) infected with an adenoviral vector containing the BMP7 gene have been shown to improve femur regeneration in goats (Zhu et al. 2010). In addition, the use of gelatin/tricalcium phosphate ceramic/glutaraldehyde biopolymer with viral vector transfer of the BMP gene can significantly improve bone healing (Chang et al. 2009; Kadiyala et al. 1997). In addition, there is also evidence of improved healing of calvarial bone defects by implantation of Osx-transduced bone marrow mesenchymal stem cells BMSC resulted in 85% healing of calvarial bone defects as detected by radiological analysis. While challenges remain in ensuring the safety, efficacy, and ethical application of these therapies, gene-based interventions hold immense potential for revolutionizing treatments for fractures, bone defects, and degenerative bone diseases. By leveraging natural processes and enhancing them with genetic tools, bone regeneration can be significantly accelerated, ensuring better outcomes for patients and advancing the field of regenerative medicine.

5.2 Cardiovascular regeneration

Regeneration of cardiovascular tissue is a complex and multi-stage process, including restoration of the structures and functions of the cardiovascular system. The main approaches to cardiovascular tissue regeneration are the use of growth factors, stem cells, gene therapy, and microRNA-based interventions to promote cardiac tissue repair and regeneration. For effective repair of cardiac tissue, a reliable blood supply is necessary. Gene therapy can stimulate angiogenesis, the formation of new blood vessels. Vascular endothelial growth factor (VEGF) plays a critical role in angiogenesis, the formation of new blood vessels from pre-existing ones. VEGF has been shown to stimulate endothelial cell chemotaxis and stimulate the formation of new vessels. This ability to promote the growth of new vasculature makes VEGF an important factor in the regeneration and repair of the cardiovascular system (Nissen et al. 1998; Kim et al. 2004). Notably, the hearts of 1-day-old newborn mice can regenerate after partial surgical resection, and this ability is lost by 7 days of age. This process differs from conventional regeneration processes as it is not associated with fibrosis and hypertrophy (Porrello et al. 2011). The adult mammalian heart has a limited ability to renew itself from pre-existing cardiomyocytes. Cardiomyocytes turn over, although at a decreasing rate with age: turnover is 1% annually at age 25 and declines to 0.45% by age 75 (Bergmann et al. 2009). To stimulate cardiomyocyte regeneration we can, for example, induce re-entry into the cell cycle. Thus, delivery of the cyclin A2 gene (Ccna2) to the hearts of pigs subjected to infarction stimulates regeneration, as evidenced by an increase in the number of cardiomyocytes and a decrease in fibrosis. Moreover, administration of nonintegrating retroviruses encoding Cdk1/CyclinB1 and Cdk4/CyclinD1 (TNNT2-4Fpolycystronic-NIL) effectively induces cardiomyocyte proliferation and alleviates subacute ischemic heart failure (IHF) in animal models by preventing organ stagnation (Abouleisa et al. 2024). The transcription factor GATA4 plays a key role in cardiac regeneration (Kikuchi et al. 2010). Its numbers are high immediately after birth and rapidly decrease during the first week of life, which correlates with the loss of regenerative capacity. Restoring GATA4 levels via adenoviral vectors markedly improves cardiac regeneration after injury. And therefore, GATA4 knockout leads to impaired regeneration with larger scars and decreased proliferation of cardiomyocytes (Malek Mohammadi et al. 2017). We can also reprogram postnatal cardiac or dermal fibroblasts directly into cardiomyocyte-like cells using a set of transcription factors, seemingly recapitulating

the path of cardiac development, such as Gata4, Mef2c and Tbx5. In this manner, induced cardiomyocytes express cardiac-specific markers, have gene expression profiles similar to native cardiomyocytes, and contract spontaneously, demonstrating a viable approach to generating new cardiomyocytes for cardiac repair (Ieda et al. 2010). MicroRNAs (miRNAs) have emerged as important regulators of cardiac regeneration. For example, miR-199-3p, which is responsible for myogenic differentiation and muscle regeneration. Its amount in the blood decreases with age. In turn, the administration of miR-199 to muscle fiber hypertrophy and delayed loss of muscle strength. Introduction of microRNAs into the heart stimulates cardiac regeneration through the proliferation of cardiomyocytes (Fukuoka et al. 2021). The existence of cells with cardiac stem cell properties confirms the potential for cardiac regeneration. These cells are self-renewing, clonogenic and multipotent, capable of differentiating into myocytes, smooth muscle and endothelial cells. By introducing such cells into an ischemic heart, we can restore a well-differentiated myocardium with new blood vessels and young myocytes (Beltrami et al. 2003). Another possibility for cardiac regeneration is the introduction of mesenchymal stem cells (MSCs). Genetically modified, these cells can be engineered to overexpress Bcl-2, which has been demonstrated to improve viability after transplantation into the myocardium. Bcl-2 overexpression reduces MSC apoptosis and enhances VEGF secretion under hypoxic conditions, thus demonstrating improved viability after myocardial transplantation. Transplantation of Bcl-2-MSCs significantly increased cell survival, capillary density, and functional recovery, and reduced infarct size compared with control MSCs (Wenzhong Li et al. 2007). Cardiovascular regeneration gene therapy holds immense potential to revolutionize the treatment of heart and vascular diseases by addressing the underlying causes of tissue damage and loss. While challenges remain, ongoing research continues to refine and expand these therapies, offering hope for millions of patients worldwide who suffer from debilitating cardiovascular conditions.

5.3 Liver regeneration

The liver is known for its remarkable regenerative ability, which allows it to recover from damage and restore its functions. This process can be greatly accelerated by gene therapy, which involves introducing or modifying genes in liver cells to promote healing and regeneration after injury. Liver regeneration is a complex, well-controlled process. The transition of fully differentiated hepatocytes from the G0 phase (a resting or quiescent state) to the G1 phase (the first phase of the cell cycle) is a pivotal step in liver regeneration. This transition marks the reactivation of the cell cycle in mature liver cells, allowing them to proliferate and replace lost or damaged tissue. This process is tightly regulated by signaling pathways, growth factors, and cytokines, ensuring efficient and controlled liver repair. This is facilitated by liver cells and several signaling molecules, such as Wnt protein (Monga et al. 2001), hepatocyte growth factor (HGF) and the cytokines TNFα (FitzGerald et al. 1995), IL-6 (Cressman et al. 1996; Iimuro et al. 1998; Kirillova, Chaisson, and Fausto 1999). They stimulate several signaling cascades that ultimately lead to hepatocyte proliferation (Fig. 4). Strategies for gene therapy can be different, such as stimulating proliferation or inhibiting apoptosis. Studies in mice show that intravenous injection of recombinant human HGF (rhHGF) stimulates DNA synthesis and increases the number of hepatocytes, with the effect being dose dependent (Phaneuf, Chen, and

Wilson 2000). The effectiveness of using HGF as a treatment is also supported by the acceleration of liver recovery after cirrhosis in mice and other animal models (Xue et al. 2003; Horiguchi et al. 2009). Among other things, the introduction of the HGF gene reduced hepatocyte apoptosis in rats after massive hepatectomy (Yuasa et al. 2007). An alternative option is *ex vivo* gene therapy, which involves obtaining hepatocyte-like cells from reprogrammed somatic or stem cells and further integrating them into the body (K.-D. Lee et al. 2004; Y.-F. Chen et al. 2012). Liver regeneration gene therapy has the potential in the treatment of liver diseases by addressing the root causes of impaired regeneration. Advances in vector technology, gene editing tools, and the understanding of liver biology are paving the way for innovative therapies that can restore liver function, reduce fibrosis, and improve outcomes in both acute and chronic liver diseases.

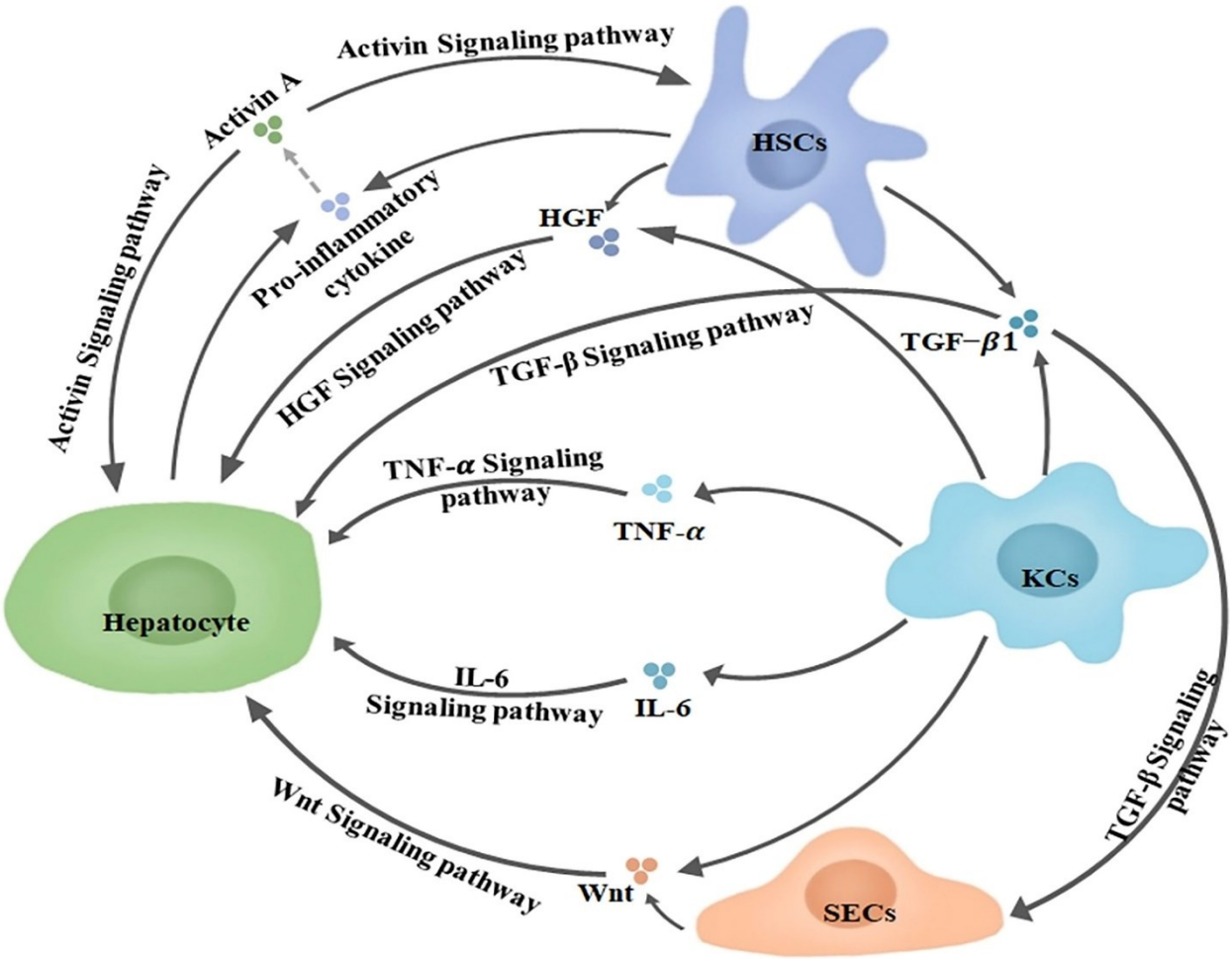

Figure 4. The main activated signaling pathways in liver regeneration

KCs and HSCs secrete TGFβ1 and activate TGF-β signaling pathway to act on SECs and hepatocytes. KCs and HSCs secrete HGF and activate HGF Signaling pathway to act on hepatocytes. KCs secrete TNF-α, IL-6 and activate TNF-***α*** and IL-6 signaling pathway to act on hepatocytes. KCs and SECs secrete Wnt and activate Wnt signaling pathway to act on hepatocytes. HSCs and hepatocytes secrete pro-inflammatory cytokine activating activin A and activin signaling pathway that acts on HSCs and hepatocytes. HSCs, Hepatic stellate cells; KCs, Kupffer cells; SECs, Hepatic sinusoidal endothelial cells; HGF, Hepatocyte growth factor; TG.F-β, Transforming growth factor β; TNF-α, Tumor necrosis factor-α; IL-6, Interleukin-6. Dotted line arrow represents activation between cytokines (C. Zhang et al. 2024).

5.4 Neural regeneration

The nervous system is a complex network of neurons and supporting cells that transmit signals between different parts of the body. It is responsible for coordinating and controlling body functions, processing sensory information, and facilitating cognition, emotion, and behavior. The nervous system is divided into two main parts: the central nervous system (CNS) and the peripheral nervous system (PNS). Nerve regeneration is the process by which neurons are repaired or regenerated to restore their function after injury or disease. Regeneration of the CNS is a complex task that requires a multifaceted approach to overcome internal and external barriers. Unlike in the PNS, where regeneration naturally follows injury, unfortunately, the adult mammalian CNS lacks the ability to regenerate after injury. However, research in the field of CNS regeneration very promising. Gene therapy here aims to deliver therapeutic genes to affected brain regions to promote neuroprotection, neuroregeneration, angiogenesis and functional recovery. It is an important area of research in neuroscience and medicine because of its potential for treating conditions such as spinal cord injury, neurodegenerative diseases (such as Alzheimer's and Parkinson's disease) and stroke. Neuroprotection refers to interventions aimed at preserving the structure and function of neurons due to various injuries, for example, caused by neurodegenerative diseases, traumatic injuries or strokes. The goal is to prevent neuronal loss and preserve nerve function. Various approaches can be used for neuroprotection, including Bcl-2 apoptosis inhibitors, which promote axonal regeneration (D. F. Chen et al. 1997) and anti-inflammatory cytokines, which reduce the expression of pro-inflammatory genes and increased pro-regenerative genes (Park et al. 2018), suppress activation and proliferation T cells, which in turn helps prevent excessive immune activation and the release of pro-inflammatory factors that can contribute to secondary damage (Haro et al. 2023). Neuroregeneration, which promotes the growth and repair of damaged neurons and axons, usually involves the expression of neurotrophic factors, for example, BDNF, NT-3, GDNF. For example, expression of BDNF, NT-3 enhanced propriospinal axonal regeneration and, more importantly, promoted axonal regeneration of certain distant populations of brainstem neurons into midthoracic grafts in the adult rat spinal cord (X. M. Xu et al. 1995). GDNF increases both the number and caliber of regenerated axons *in vivo*, and also increased neurite outgrowth of dorsal root ganglion neurons (DRGN) *ex vivo*, suggesting that GDNF has a direct effect on neurons (L. Zhang et al. 2009). Angiogenesis is required to improve blood supply to the damaged spinal cord, necessary for tissue restoration. Vascular endothelial growth factor (VEGF) enhances angiogenesis and improves tissue oxygenation and nutrient supply. Finally, functional recovery may be facilitated by a transcription factor SOX2 that can reprogram astrocytes into neurons *in vivo* (Su et al. 2014). Neural regeneration gene therapy offers immense potential to address the challenges of repairing and restoring function in the nervous system. By harnessing growth factors, neuroprotective molecules, and advanced delivery systems, this approach is paving the way for breakthroughs in treating spinal cord injuries, neurodegenerative diseases, and other neural conditions. Ongoing research into optimizing delivery methods, overcoming inhibitory factors, and enhancing safety profiles will likely make these therapies a cornerstone of regenerative neurology in the future.

6 Preclinical and Clinical Studies

Preclinical trials serve as the foundational step in developing gene therapies, involving rigorous testing in vitro (in cell cultures) and *in vivo* (in animal models). The objectives are to demonstrate proof of concept, optimize delivery mechanisms, and evaluate the safety and efficacy of the therapeutic gene. Trials can be conducted both *in vivo* and *ex vivo*. *Ex vivo* experiments assess gene expression, stability, and functionality in cultured cells derived from the targeted tissue. For regenerative medicine, cells like mesenchymal stem cells (MSCs) or induced pluripotent stem cells (iPSCs) are commonly used. *In vivo* studies use animal models, that are crucial for evaluating how the therapy performs in a living organism. Preclinical studies focus on biodistribution toxicity and therapeutic efficacy. These studies must adhere to Good Laboratory Practices (GLP) and meet regulatory requirements (e.g., FDA, EMA) before advancing to clinical trials. Once preclinical success is established, the therapy progresses to clinical trials, conducted in phases to assess safety, dosage, efficacy, and long-term effects in humans. The use of gene therapy in regenerative medicine is still a relatively new direction. In this regard, most of the studies are in the preclinical phase. However, despite this, this area is also actively developing. Today, there are already several hundreds of clinical trials in one phase or another. Below are some of them (tab. 5). In addition, there are already approved commercial products like Spinraza (for treatment of Spinal Muscular Atrophy; EMA product number: EMEA/H/C/004312). Preclinical and clinical trials are pivotal in advancing gene therapy applications within regenerative medicine. While challenges remain, the rapid evolution of gene-editing tools, delivery systems, and ethical frameworks holds promise for a future where regenerative medicine transforms healthcare.

Table 5. Clinical trials of gene therapy in regenerative medicine,

	Condition	**Target gene and/or pathway**	**Metod/Vector**	***Ex vivo* or *in vivo***	**Phase**	**ClinicalTrials.gov ID**
Muscle Regeneration	BMD	*Follistatin*	AAV	*In vivo*	Phase 1	NCT01519349
	DMD	*Dystrophin*	AAV	*In vivo*	Phase 1	NCT06114056, NCT03362502
			snRNA/AAV	*In vivo*	Phase 1/2	NCT04240314
			ASO/ IV injection	*In vivo*	Phase 1/2	NCT06280209, NCT04906460
					Phase 3	NCT03907072
			CRISPR/Cas9/AVV	*In vivo*	Early Phase 1	NCT06392724
		Follistatin	AAV	*In vivo*	Phase 1/2	NCT02354781
		GALGT2	AAV	*In vivo*	Phase 1/2	NCT03333590
		H-μD5	AAV	*In vivo*	Phase 1/2	NCT06138639
	LGMD	*β-SG*	AAV	*In vivo*	Phase 3	NCT06246513
Cardiovascular Regeneration	DM1	*MBNL*/miR-23b	ASO/injection	*In vivo*	Phase 1/2	NCT06300307
	Severe coronary heart disease	*VEGF-D*	AdV	*In vivo*	Phase 1	NCT01002430

	Critical limb ischemia	*HGF*	Plasmid/ IM injection	*In vivo*	Phase 3	NCT04274049
	Congestive Heart Failure	*I-1c*	AAV	*In vivo*	Phase 2	NCT05598333, NCT04179643
		ADCY6	AAV	*In vivo*	Phase 1/2	NCT00787059
	DMD-Associated Dilated Cardiomyopathy	*SERCA2a*	AAV	*In vivo*	Phase 1	NCT06224660
	Heart Failure	*SERCA2a*	AAV	*In vivo*	Phase 1	NCT06061549
	Cardiomyopathy of Friedreich's Ataxia	*Frataxin*	AAV	*In vivo*	Phase 1	NCT05302271
Neural Regeneration	ALS	*Calpain-2*	ASO/ IT injection	*In vivo*	Phase 1	NCT06665165
		SOD1	SiRNA/ IT injection	*In vivo*	Phase 1	NCT06351592
	Alzheimer's Disease	*APOE4*	AAV	*In vivo*	Phase 1	NCT05400330
		MAPT	ASO/ IT injection	*In vivo*	Phase 1	NCT06372821
		NGF	AAV	*In vivo*	Phase 2	NCT00876863
		BDNF	AAV	*In vivo*	Phase 1	NCT05040217
	Canavan disease	*ASPA*	AAV	*In vivo*	Phase 1/2	NCT04998396, NCT04833907
	FTD-GRN	*GRN*	AAV	*In vivo*	Phase 1/2	NCT04408625, NCT06064890
					Phase 1	NCT04747431
	Huntington's Disease	*CH24H*	AAV	*In vivo*	Phase 1/2	NCT05541627
	Parkinson's Disease	*GDNF*	AAV	*In vivo*	Phase 2	NCT06285643
Skin Regeneration	Dystrophic Epidermolysis Bullosa	*COL7A1*	ASO/ poloxamer	*In vivo*	Phase 1/2	NCT05529134
	RDEB	*COL7A1*	Retrovirus	*Ex vivo*	Phase 1/2	NCT04186650
Hemopoietic Stem Cell Regeneration	Fanconi Anemia	*FANCA*	Retrovirus	*Ex vivo*	Phase 1/2	NCT03157804
					Phase 2	NCT04069533
	Sickle Cell Disease	*BCL11A*	CRISPR/Cas9	*Ex vivo*	Phase 1	NCT04443907, NCT06506461
		HBB	Retrovirus	*Ex vivo*	Phase 3	NCT04293185
		βA-T87Q-globin	Retrovirus	*Ex vivo*	Phase 1/2	NCT06399107
Vision Restoration	wAMD	*Anti-VEGF*	AAV	*In vivo*	Phase 1/2	NCT05984927, NCT06458595
		Anti-VEGF	AAV	*In vivo*	Phase 3	NCT05407636
	DME	*Anti-VEGF*	AAV	*In vivo*	Phase 1	NCT06237777
					Phase 2	NCT04567550, NCT04418427

	Retinitis pigmentosa	*NR2E3*	AAV	*In vivo*	Phase 1/2	NCT05203939
	Stargardt Disease	*ABCA4*	AAV	*In vivo*	Phase 1/2	NCT06300476, NCT06467344
Liver Regeneration	Wilson's Disease	*ATP7B,*	AAV	*In vivo*	Phase 1/2	NCT04884815, NCT04537377
Cartilage Regeneration	Osteoarthritis	*Nkx3.2*	AAV	*In vivo*	Phase 1/2	NCT05454566
		IL-1Ra	AAV	*In vivo*	Phase 1	NCT05835895

AAV, Adeno-Associated Virus; *ABCA4, ATP Binding Cassette Subfamily A Member 4; ADCY6, Adenylyl Cyclase Type 6;* AdV, Adenovirus; ALS, Amyotrophic Lateral Sclerosis; *Anti-VEGF, Anti-Vascular Endothelial Growth Factor; APOE4, Apolipoprotein E;* ASO, Antisense Oligonucleotide; *ASPA, Aspartoacylase; ATP7B, ATPase Copper Transporting Beta; BCL11A, BCL11 Transcription Factor A; BDNF, Brain-Derived Neurotrophic Factor;* BMD, Becker Muscular Dystrophy; *CH24H, Cholesterol 24-hydroxylase;* CMD, Congenital Muscular Dystrophy; *COL7A1, Collagen Type VII Alpha 1 Chain; DM1, Myotonic Dystrophy Type 1;* DMD, Duchenne Muscular Dystrophy; DME, Diabetic Macular Edema; *FANCA, FA Complementation Group; FTD-GRN, Frontotemporal Dementia with Progranulin Mutations; GALGT2, β-1,4-N-Acetylgalactosaminyltransferase 2; GDNF, Glial Cell Line-derived Neurotrophic Factor; GRN, Granulin Precursor; h-μD5, Human Microdystrophin Gene; HBB, Hemoglobin Subunit Beta; HGF, Hepatocyte Growth Factor; I-1c, Inhibitor-1; IL-1Ra, IL-1 Receptor Antagonist;* IM, Intramuscular; IT, Intrathecal; IV, Intravenous; LGMD, Limb-Girdle Muscular Dystrophy; *MAPT, Microtubule-associated Protein Tau; MBNL, Muscleblind Like Splicing Regulator; NGF, Nerve Growth Factor; Nkx3.2, NK3 Homeobox 1; NR2E3, Nuclear Receptor Subfamily 2 Group E Member 3;* RDEB, Recessive Dystrophic Epidermolysis Bullosa; *SERCA2a, Sarcoplasmic/endoplasmic reticulum Ca(2+) ATPase 2a;* SMA, Spinal Muscular Atrophy; snRNA, Small Nuclear RNA; *SOD1, Superoxide Dismutase-1; VEGF-D, Vascular endothelial growth factor-D;* wAMD, Wet Age-related Macular Degeneration; *β-SG, Beta-sarcoglycan.*

7 Future Perspectives and Challenges

Gene therapy holds tremendous promise in the field of regenerative medicine, with the potential to cure or alleviate various diseases and conditions that involve tissue damage, genetic disorders, and organ dysfunction. By modifying the genetic material in a patient's cells, gene therapy can address the root causes of disease, offering potential for long-term healing and regeneration of tissues or even organs. However, despite its potential, gene therapy in regenerative medicine faces a number of challenges and limitations that need to be overcome to utilize its full benefits. One of the areas for development is personalized medicine. As gene therapy becomes more refined, it will play a significant role in personalized medicine, where therapies can be tailored to an individual's unique genetic makeup. This could enhance the effectiveness of treatments and minimize side effects. One of the most exciting prospects is the use of gene therapy to regenerate complex tissues such as the heart, liver, and neurons that have limited regenerative capacity. By stimulating specific genes that drive stem cell differentiation and tissue growth, gene therapy could enable the restoration of damaged or aged organs. The possibility of using gene therapy in combination with stem cells could enable the creation of fully functional organ tissues, potentially reducing the need for organ transplants.

One of the most significant hurdles is the immune response triggered by the introduction of foreign DNA, particularly when viral vectors are used to deliver the gene therapy. Patients' immune systems may recognize these vectors as foreign and mount an immune response, potentially leading to side effects or failure of the therapy. Developing more immunologically "stealth" gene delivery systems or using self-expressing vectors is crucial to reduce the risk of immune rejection. Achieving efficient and targeted delivery of therapeutic genes also remains a major challenge. Most gene therapies rely on viral vectors to deliver genetic material into a patient’s cells, but these vectors cannot always target the desired cells or tissues. This have led to a series of experiments that succeeded in creating a chimeric virus, namely AAV. Such chimeric viruses are unique and do not occur in nature, and therefore humans will not have immunity to them. In this way we can avoid unwanted inflammatory reactions and neutralization of the viral vector. In addition, such vectors can change tropism to various tissues, such as skeletal muscle, liver and brain in mice and nonhuman primates (Wuping Li et al. 2008). Further possible improvements to viral vectors may be rational design of the capsid. This technique has demonstrated improvement in transgene expression, for example, in the retina (Petrs-Silva et al. 2009) and CNS (Kanaan et al. 2017) in animal models and human cells *ex vivo* (Aslanidi et al. 2013). Other components of gene therapy are also amenable to rational design, for example Crispr/Cas9. This will help us reduce the likelihood of off targeted editing and, as a consequence, potential unwanted mutations in the genome (Kleinstiver et al. 2016; Slaymaker et al. 2016; J. S. Chen et al. 2017). The long-term effects of gene therapy are still largely unknown. While gene editing offers the potential for permanent corrections of genetic defects, there is uncertainty about how these modifications will behave over time. Will they continue to function as expected in the long run? Are there risks of unforeseen consequences, such as oncogenesis? Thorough long-term clinical trials and monitoring will be needed to assess the safety and durability of gene therapies for regenerative applications. Currently, gene therapy is very expensive, which limits its accessibility to a broad population. Manufacturing gene therapies at scale, improving cost-effectiveness, and ensuring

equitable access will be crucial for widespread adoption. Gene therapy has the potential to revolutionize regenerative medicine by offering treatments that can correct genetic defects, regenerate tissues, and restore organ function. However, significant challenges remain, particularly related to the safe and efficient delivery of genes, immune responses, long-term safety, and cost-effectiveness. Continued technological innovation, rigorous clinical testing, and regulatory advancements will be essential to overcoming these challenges and realizing the full potential of gene therapy in regenerative medicine. As the field evolves, it holds the promise of transforming how we approach treatment for a wide range of diseases, from genetic disorders to degenerative diseases and beyond.

8 Conclusion

Gene therapy in regenerative medicine stands at the forefront of innovation, offering transformative solutions for repairing and replacing damaged tissues and organs. By leveraging advanced techniques such as gene editing, viral and non-viral vectors, and cellular reprogramming, gene therapy has demonstrated significant potential to address genetic disorders, stimulate tissue regeneration, and enhance the body's healing processes. Despite remarkable advancements, challenges such as delivery efficiency, immune responses, long-term safety, and ethical considerations must be overcome to fully integrate these therapies into clinical practice. Continued research and technological progress hold promise for realizing the full potential of gene therapy, paving the way for revolutionary treatments in regenerative medicine. The combination of gene therapy with stem cell technologies opens up new avenues for personalized treatments and long-term solutions. By directly influencing the genetic makeup of cells, it is possible to stimulate regeneration, promote tissue repair, and correct underlying genetic disorders. However, challenges related to safety, efficiency, delivery methods, and ethical considerations remain. Preclinical and clinical studies are essential in refining these therapies, ensuring their effectiveness, and addressing potential risks. As research progresses, the application of gene therapy in regenerative medicine is expected to grow, offering hope for patients who currently have limited treatment options. By overcoming existing barriers and improving the scalability and accessibility of these therapies, gene therapy can become a cornerstone of future regenerative medicine practices, potentially transforming the landscape of healthcare.

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