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The Interface of Gene Editing with Regenerative Medicine

Veronica E. Farag[a], **Elsie A. Devey**[a], **Kam W. Leong**[a,b,*]

[a]Department of Biomedical Engineering, Columbia University, New York City, NY 10027, USA

[b]Department of Systems Biology, Columbia University, New York City, NY 10032, USA

Abstract

The potential of regenerative medicine in the clinical space is vast, given its ability to repair and replace damaged tissues, restore lost functions due to age or disease, and transform personalized therapy. Traditional regenerative medicine and tissue engineering strategies have created specialized tissues using progenitor cells and various biological stimuli. To date, there are many US Food and Drug Administration (FDA)-approved regenerative medicine therapies, such as those for wound healing and orthopedic injuries. Nonetheless, these therapies face challenges, including off-target effects, a lack of precision, and failure to target the disease or injury at its origin. In search of novel, precise, and efficient alternatives, the regenerative medicine landscape is shifting towards genome engineering technologies, particularly gene editing. Clustered regularly interspaced short palindromic repeats (CRISPR)-based gene editing systems enable precise knock-ins, knockouts, transcriptional activation and repression, as well as specific base conversions. This advancement has allowed researchers to treat genetic and degenerative diseases, control cell fate for highly regulated tissue repair, and enhance tissue functions. In this review, we explore the progress and future prospects of CRISPR technologies in regenerative medicine, focusing on how gene editing has led to advanced therapeutic applications and served as a versatile research tool for understanding tissue development and disease progression.

Keywords

CRISPR/Cas9; Tissue engineering; Regenerative medicine; Gene editing; Stem cell transplantation; Disease models

1. Introduction

Both the development of induced pluripotent stem cells (iPSCs) from mature somatic cells and clustered regularly interspaced short palindromic repeats (CRISPR) with CRISPR-associated (Cas) proteins have won Nobel Prizes and led to breakthroughs in their respective fields. When used independently, iPSCs have the potential to regenerate damaged, injured, or depleted tissue, while CRISPR/Cas9 can efficiently alter the genome for medicinal, agricultural, and animal breeding applications [1]. The intersection of these technologies lies

*Corresponding author. kam.leong@columbia.edu (K.W. Leong).

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in the principle that gene expression governs both the deterioration and repair of tissues. Thus, hijacking gene expression is a plausible option for creating disease models as well as for tissue regeneration. In this review, we broadly define regenerative medicine as the repair of mutated, damaged, injured, or deficient tissues. Traditional regenerative medicine strategies rely on embedding stem cells in biocompatible scaffolds or using transcriptional gene delivery to stimulate cell differentiation into the desired tissue. While these strategies have had some success, they are often limited to producing transient benefits due to low *in vivo* stability, failing to target the underlying causes of tissue damage, and being potentially immunogenic. To address these challenges, we will present genome engineering, specifically CRISPR/Cas9, as an efficient, precise, and versatile therapeutic approach for regenerative medicine. Moreover, we will discuss the application of gene editing as a potent research tool that supports mechanistic studies in this field.

For therapeutic applications, CRISPR technologies have been used to correct genetic diseases, reprogram cells to compensate for lost or deficient tissue, evade allograft immune rejection, and precisely control cellular protein dosage—all of which contribute to restoring tissue to its original state. Additionally, CRISPR technologies have been employed for functional genomics and disease modeling, proving to be valuable tools in regenerative medicine applications and translational research. This review will explore the current state of CRISPR technologies in regenerative medicine, highlighting both their constraints and prospects for future advancements.

2. Tissue engineering and regenerative medicine

Regenerative medicine restores lost functions of damaged tissues and organs. Often intertwined with regenerative medicine, and a subset of the field is tissue engineering which manufactures structures that mimic, maintain, or restore the normal ability of tissues [2]. Specifically, the transplantation of stem cells within biomaterial scaffolds and frequently with biologically active molecules can promote tissue regeneration [3]. Tissue engineering and regenerative medicine leverage the self-renewal of stem cells to restore lost functions and biomaterial scaffolds to mimic the extracellular matrix (ECM) and circumvent immunogenicity. These strategies offer a promising alternative to organ transplantation, given the limited number of donors and the frequency of immune rejection. This strategy has been applied to neural, bone, and renal regeneration, with some engineered tissues such as skin and cartilage having Food and Drug Administration (FDA) approval [3,4]. Despite challenges of immunogenicity and other limitations of tissue engineering materials, this growing multidisciplinary field has brought about several promising advances for treating complex, chronic diseases.

2.1. Advances and progress in tissue engineering

The success of tissue-engineered therapies lies in their ability to efficiently and effectively regenerate lost tissue. This success is bolstered by novel technologies that strengthen the existing tissue engineering toolbox. Developments such as smart biomaterials, enhanced bioreactors, advanced three dimensional (3D) printing, and new stem cell sources have all contributed to the therapeutic potential of engineered tissues [5]. For instance, the

use of decellularized ECM-based scaffolds in tissue-engineered products creates ideal stem cell environments for growth and differentiation, given that these cells are growing in an environment similar to their original setting. SynerGraft (CryoLife, USA), a decellularized pulmonary allograft has been shown to have lower immunogenicity compared to conventional allografts [6]. Additionally, the exploration of unique material properties has expanded the potential of tissue-engineered products. Synthetic hydrogels, for example, are notable for their water retention properties, making them ideal candidates for kidney regeneration applications [4]. Hydrogels broadly offer a biomimetic environment for cell embedding and growth and can be tuned to promote tissue regeneration by adjusting their porosity, composition, and elasticity [7].

Furthermore, advancements in multi-material 3D bioprinting have facilitated the creation of microchannels within tissue constructs, enhancing vascularization and the diffusion of oxygen and nutrients, and allowing specialized cell organization [8]. Efforts have successfully embedded differentiated cardiomyocytes and epithelial cells onto 3D-printed collagen scaffolds and hydrogels—mitigating challenges posed by nonproliferative cardiomyocytes [9,10]. The resulting constructs geometrically mimic the complex structure of the heart and have a porous microstructure that allows for vascularization and cell infiltration [9,10]. Advanced printing techniques are particularly advantageous when mimicking the complex geometries of human tissues and organs. To successfully recapitulate the rete ridges between the epidermal and dermal layers, researchers relied on 3D printed stamps and microfolding, ultimately creating patterns with controlled geometry and periodicity [11]. Four dimension (4D) bioprinting enables constructs to change their shape and properties in response to stimuli, such as specialized cell differentiation following electrical stimulation [12,13].

Embryonic stem cells (ESCs), with their pluripotency and capacity for differentiation, are highly valued in tissue engineering, despite ethical controversies. Significant efforts have been made to identify alternative stem cell sources, such as those derived from the placenta or amniotic fluid, or through reprogramming somatic cells into iPSCs. To replicate the multicellular structure of tissue constructs like neural tissue using iPSCs, researchers have successfully employed an orthogonal differentiation method [14]. Instead of relying on media cues, they used transcription factors to induce differentiation [14].

These advancements have propelled tissue-engineered products toward clinical translation and FDA approval. Various engineered tissues, including cartilage, bone, skin, bladder, vascular grafts, trachea, and cardiac tissues, have been used in patients [15]. Between 2008 and 2021, most clinical trials in the European Union (EU) for tissue-engineered products targeted musculoskeletal diseases, cardiovascular diseases, and skin/connective tissue diseases [16]. Marketed products such as OrCel (Forticell Bioscience, USA), which uses human fibroblasts on bovine collagen for the treatment of burn wounds, and Dermagraft (Organogenesis, USA) which uses human fibroblasts on a poly(lactic-*co*-glycolic acid) (PLGA) scaffold for diabetic foot ulcer repair [17], exemplify successful applications. However, despite these advances, regenerating complex tissues such as the heart and lungs remains a formidable challenge. *In vitro*, tissue engineering technologies can also be used

for disease modeling, drug screening, and drug development, particularly with organoids or organ-on-a-chip technology.

2.2. Limitations and challenges in tissue engineering

Despite significant progress in the fields of tissue engineering and regenerative medicine, there is still ample space for further advancements. Traditional tissue-engineered therapies have pre-dominantly relied on autologous stem cells to circumvent immune rejection. In fact, in a study analyzing trends of tissue-engineered products in clinical trials in the EU, over half of the studies rely on autologous cell sources and it was found that autologous products are in later phases of development than allogeneic cell products defining an important correlation between cell source and trial phase [16]. Despite this fact, in recent years there has been an increase in the number of trials relying on allogeneic sources, given that autologous cell products suffer from low manufacturing capacities and high variabilities in the final therapeutic product [16]. Moreover, current research is increasingly focused on developing broadly immunocompatible and off-the-shelf tissue-engineered products. Nonetheless, the sourcing of allogeneic stem cells presents its own set of challenges, including ethical considerations, high costs, regulatory barriers, and safety concerns. Gene editing offers a promising avenue by facilitating the generation of iPSCs and various specialized cell types, crucial for constructing complex tissues that require a diversity of cell types, thereby addressing these concerns. Moreover, in Section 4.3, we will delve into how advancements in gene editing for regenerative medicine could address the challenge of creating non-immunogenic products, though it’s important to note that this approach primarily addresses the immunogenicity of the cellular component and not the scaffold material.

The shelf-life of tissue-engineered products is short, with most products unable to exceed four days due to the complexity of preserving 3D structures while maintaining cell viability [16]. Many successful techniques for preserving isolated cells, such as slow freezing or dry state preservation, are ineffective when applied to tissue-engineered products [18]. This necessitates new methods to address both preserved and on-demand products to facilitate clinical translation and reduce patient wait times. One study found that 48-hour preservation in a hypothermic (4 °C) phosphate-buffered saline solution maintained the viability and integrity of tissue-engineered bone from human iPSCs, which was not achieved with cryopreservation medium [19]. In another study, researchers successfully preserved human adipose stromal/stem cell sheet-like confluent cultures in preservation solutions at hypothermic (4 °C) temperatures, observing retained metabolic activity, preserved ECM integrity, and maintained adipogenic and osteogenic differentiation [20]. However, the success of hypothermic preservation methods does not address long-term preservation concerns. This highlights the need for robust and rapid sterility methods, as well as cell banking with versatile donor databases, to create on-demand tissue-engineered products if long-term preservation techniques prove to be infeasible.

The success of a tissue-engineered product is limited by its ability to vascularize and innervate upon implantation, *in vivo*, to ensure effective integration. Some researchers have successfully mimicked the high-density cell composition and alignment of cardiac

tissue, allowing for control over the magnitude and direction of contractile forces [21]. Nonetheless, the translational potential of this tissue is limited by its lack of vasculature. Conversely, other researchers have successfully 3D printed thick, vascularized cardiac tissue, but it is limited by its ability to pump blood, low cell density, and capacity for printing small vessels [22]. Recent work has found that adipose tissue-derived microvascular fragments (ad-MVFs) are promising vascularization units due to their intrinsic angiogenic potential. One study found that 24-hour cultivation at subnormothermic (20 °C) temperatures enhanced *in vivo* vascularization with a higher density of microvessels compared to normothermically (37 °C) cultivated ad-MVFs and noncultivated controls [23]. While MVF-based vascularization strategies hold promise, future work is needed for both their optimization and scalability, given that isolated human ad-MVFs have lower viability compared to animal ad-MVFs [24]. Overall, while the field has succeeded in creating simplified tissue models that have been effective in many applications, recreating the full complexity of organs at both cellular and tissue levels remains elusive. Overcoming this challenge will depend on the advancement of fabrication technologies, improving their scalability, precision, and consistency.

Given the distinct characteristics and requirements of each tissue or organ, tissue-engineered therapies demand a tailored approach. This necessitates careful consideration of various factors, including cell type selection, ECM interactions, the influence of physical forces, dynamic effects, and spatial constraints, to successfully develop effective therapies.

3. Gene editing

The hallmark of gene editing is through CRISPR and its associated endonuclease, which is inspired by the prokaryotic immune system [25]. CRISPR/Cas9 stands out among all other gene editors, such as zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) for its simplicity, specificity, and reproducibility [26].

3.1. Basics of CRISPR/Cas9 editing

CRISPR/Cas9 relies on an endonuclease as well as a guide sequence to orient the nuclease to its target site. Upon detecting its site of action, catalytically active endonucleases induce a double-strand break (DSB). Cells subsequently respond with two different DNA repair mechanisms: non-homologous end joining (NHEJ) or homology-directed repair (HDR). The former generates insertions or deletions (indels) at the cutting site and the latter requires donor templates with sequence-sufficient homology to integrate at the cutting site (Figs. 1(a) and (b)). NHEJ typically creates knockout or knock-in models at the cutting site, while HDR creates controlled and precise knock-in models [27]. However, the edits produced by NHEJ are variable in length and difficult to predict even using computational algorithms. Thus, the indels created by NHEJ may not be sufficient to create the intended edits, thus limiting the capacity for mutations NHEJ can correct. Moreover, HDR may not be favored due to its inefficiency and infrequency accredited to its cell cycle limitations [28]. HDR is inactive in non-cycling cells or during the G1 phase of the cell cycle [29]. The DSBs induced by CRISPR/Cas9 are not flawless, as they have the potential to lead to chromosomal translocations, and large deletions, and can activate the p53 pathway in human stem and

progenitor cells (hSPCs) [30]. Thus, we will also explore other CRISPR methods that do not rely on DSBs for editing.

The success of a Cas endonuclease is limited in part by the presence of a compatible protospacer adjacent motif (PAM) on the complementary strand to where the single guide RNA (sgRNA) will bind. The most commonly used Cas9 homolog is the *Streptococcus pyogenes* Cas9 (SpCas9) due to the broad targeting range of its associated PAM sequence. Even with this widely found NGG PAM site, there remain genes that are non-targetable. To mitigate this, researchers have developed variants of SpCas9, such as SpG and SpRY, that are capable of recognizing an extended number of PAM sequences throughout the genome [31]. Similarly, SpCas12a also induces DNA cuts while recognizing an alternate PAM sequence. SpCas12a is unique from SpCas9 in its ability to create staggered cuts, as opposed to cuts with blunt ends. Moreover, novel Cas effectors have demonstrated transcriptional inhibition properties. Cas13 which is known for its single-strand RNA cleavage capabilities has demonstrated efficient inhibition using Cas13a, Cas13b, and Cas13d in combination with CRISPR RNA (crRNA) [32]. This technology allows for RNA knockouts that are not permanent in the genome [32].

The sgRNA is composed of crRNA and trans-activating CRISPR RNA (tracrRNA) and it is responsible for orienting the Cas9 to its target site. Moreover, the sgRNA is responsible for both CRISPR specificity (how well it targets the target site) and efficiency (how well it generates DSBs) [33]. The sgRNA can be generated using one of three methods: using plasmid DNA (pDNA), *in vitro* transcription, and chemical synthesis. Using pDNA for sgRNA generation is time-consuming and is prone to creating off-target effects and risks genomic integration, and *in vitro* transcription is experimentally challenging and prone to error. Synthetic sgRNA stands out for its improved editing efficiency and minimal risk. The sgRNA can titrate gene expression, which is important because cells exhibit specific behaviors only at gene-specific expression levels. This has implications for understanding biochemical pathways and for identifying thresholds of gene products relevant to diseases and treatments [34]. This was done by creating a sgRNA library of mismatched sgRNA activity [34]. Moreover, multiple sgRNAs can target distinct regions of the genome and fine-tune the genes' expression levels.

The most critical feature of the sgRNA is its ability to efficiently bind to its target site within the genome as this is what guides the efficacy of the endonuclease. Cas9-dependent off-target effects are generally caused by mismatches between the sgRNA and the target sequence. These effects can be minimized by carefully selecting sgRNAs through experimental evaluation, which is time-consuming. Many researchers have explored deep learning models to predict sgRNA efficiencies [35,36]. However, these models are generally limited by a lack of experimental design consistency from the studies the data are pulled from and differences in the parameters used for validating the models [35].

3.2. CRISPR systems

In addition to the generic CRISPR/Cas9 system, various new technologies are being studied. These include nickases, deactivated Cas9 (dCas9), base editors, and prime editors, all having differing editing properties.

3.2.1. Nickases: CRISPR nickases are a modified form of the generic CRISPR/Cas9 which have a mutation in one of the two nuclease domains. Thus, these enzymes create a single-stranded break in either DNA strand, rather than a DSB, depending on whether the mutation is in the RuvC or HNH domain [25]. Nickases generally minimize off-target mutagenesis, have fewer chromosomal translocations, and overall have a higher safety profile [37]. Moreover, dual Cas9 nickases can be used to create a DSB, rather than through a single Cas9 nuclease. This strategy has been shown to have similar, and sometimes higher, on-target editing efficiencies than nucleases [37–40].

3.2.2. Deactivated Cas9: Catalytically inactive dCas9 is a variant of the Cas9 enzyme. dCas9 lacks any endonucleolytic activity preventing it from forming DSBs or single-strand breaks, yet it retains its capacity to bind to DNA [41]. This strategy bypasses the cellular toxicity associated with DSBs [42]. Transcription activator or repressor domains are fused to the termini of dCas9, giving these enzymes reprogramming capacity at the transcriptional level (Fig. 1(c)). These properties label CRISPR technologies using dCas9 as CRISPR activation (CRISPRa) or CRISPR inhibition (CRISPRi) [43]. Though alternatives exist for transcriptional regulation, such as RNA interference (RNAi) or small interfering RNA (siRNA), the targeting scope of CRISPRa/CRISPRi technologies is much narrower, successfully preventing unwanted transcripts from the same transcriptional start site (TSS) from being regulated [44].

To enhance the efficacy of the dCas9, researchers have attempted to optimize the transcription activator and repressor domains. For CRISPRa, dCas9 may be fused with transcriptional activators such as VP64, p65, and Rta, or a combination of such activators to enhance transcriptional activity, such as the VPR system, consisting of VP64, p65, and Rta. dCas9 may also be fused with a tandem array of peptides, as in the SunTag system, or the synergistic activator mediator (SAM) system which uses dCas9-VP64 and MPH activation fusion proteins which are comprised of MCP, p65, and heat shock factor 1 [45–47]. CRISPRi follows similar strategies but with alternate repressor domains, such as KRAB or MeCP2, and DNA methylators such as DNMT3A [48]. In this review paper, we will explore the application of dCas9 to cellular reprogramming, targeted differentiation, genetic screening studies, and disease models.

3.2.3. Base editors: Base editors alter a base on a single strand of DNA without inducing a DSB (Fig. 1(d)). The cell's host DNA repair machinery can complement the base conversion on the complementary strand. Base editors are composed of a Cas nickase fused with a deaminase enzyme. There exist two classes of base editors: cytosine base editors (CBEs) and adenine base editors (ABEs). Despite their specificity, base editors are limited by their window of conversion, only allowing C•G to A•T conversions for CBEs and A•T to G•C conversions for ABEs, and they are likely to induce off-target edits and bystander nucleotide editing caused by random deamination. Nonetheless, engineered deaminases and cleavable deoxycytidine deaminase inhibitors have been designed to reduce off-target effects [49,50]. Many deep-learning models have been created to predict such off-target edits [51].

Given that the largest class of known pathogenic variants are point mutations, base editors are a powerful tool for correcting disease, specifically disease-associated point mutations

[52]. Due to its precision and less restrictive cell cycle requirements, base editing is more efficacious than editing through HDR. Moreover, given that base editing does not involve DSBs, this limits the risk of unwanted indels and large genomic rearrangements and translocations.

However, the efficacy of base editors is limited by the efficiency of their delivery. These Cas9 fused effectors with the sgRNA are sufficiently large and would require multiple viral vectors for delivery. Specifically, CBE or ABE plus the sgRNA is 6.9 kb, while the packaging limitation of commonly used viral vector, adeno-associated virus, is 4.9 kb [52].

3.2.4. Prime editors: A more precise and versatile alternative to base editing is prime editing (Fig. 1(e)). Like base editing, prime editing also does not rely on DSBs and is more efficient than HDR at creating substitutions and insertions. Unlike base editing, however, prime editing can introduce all twelve types of base-to-base conversions and small indels. Prime editing makes use of a reverse transcriptase fused to a Cas9 nickase. The reverse transcriptase writes information from the prime editing guide RNA (pegRNA) into the target editing region, thus replacing the original DNA sequence. The pegRNA both guides the catalytically impaired Cas9 to the target site and encodes the desired edits. Moreover, the three different DNA binding events during prime editing limit the risk of off-target editing in comparison to Cas9 nucleases. In addition, prime editing has greater editing flexibility than Cas9 nuclease by being able to edit bases farther away from the PAM site. Finally, unlike HDR, prime editing can introduce an edit at any phase of the cell cycle and does not rely on a donor DNA template.

One study used prime editing to create various base-to-base conversions and targeted indels to treat disease and insert genetic tags and epitopes [53]. Prime editing was found to have similar, or higher, efficiency to HDR, less off-target editing than Cas9 nucleases, and similar base-editing efficiencies as traditional base editors [53]. Researchers can assess the most efficient nickase variant, which one study found to be reliant on introducing additional mutations to the appropriate nuclease domain [54]. To further increase prime-editing efficacy, researchers are exploring engineered pegRNAs which have introduced mutations and structural modifications and can edit previously non-editable sites [55]. The editing specificity from using nickases with additional mutations and engineered pegRNAs in combination with the already existing versatility of prime editing paves its way as an effective tool for correcting disease and modifying the genome. Importantly, the size of these prime editing systems is maintained despite the modifications, which does not pose any additional delivery complications due to size, despite prime editors themselves being large [55].

3.3. Delivery of CRISPR machinery

Targeted and adequate delivery of CRISPR machinery is essential to proper gene editing. Here, it is critical to review delivery systems in several categories including immunogenicity, packaging capacity, genomic integration, stability, cytotoxicity, and targeting potential, among others. We have divided delivery systems into two different categories, viral and non-viral, with the latter being further divided into chemical and physical methods. Delivery

vehicles not only prevent the cargo from enzymatic degradation but also help with targeting and transport across the cell membrane. These methods are summarized in Fig. 2.

3.3.1. Delivery components: There are three different ways in which CRISPR components may be delivered: plasmid sgRNA and Cas9, Cas9 messenger RNA (mRNA) and synthetic or *in vitro* transcribed sgRNA, or a ribonucleotide (RNP) complex composed of the Cas9 protein and sgRNA. Using pDNA is inexpensive and stable, yet it has a late onset and risks both genomic integration and off-target effects. mRNA and RNPs have a lower risk for off-target effects, do not integrate within the genome, and have fast onset, but are limited by their expense and larger size, which may be difficult to deliver [56]. Moreover, RNPs no longer rely on transcription and translation to produce the editing agent and they effectively control the ratio of sgRNA to Cas9 protein.

3.3.2. Viral delivery: Viral vectors are commonly used for delivering CRISPR/Cas9 machinery *in vivo*. Specifically, many studies rely on lentiviral vectors, adeno-associated viruses (AAVs), and adenoviral vectors. Lentiviruses can hold large amounts of cargo and have low immunogenicity. However, lentiviruses integrate into the host genome, indicating there may be uncontrolled Cas9 expression caused by insertional mutagenesis, which may imply off-target editing. AAVs are characterized by their low immunogenicity, low cytotoxicity, and most notably, the fact that they do not integrate into the host genome, leading to their use in multiple clinical trials and making them a preferred method for viral CRISPR delivery. However, this vehicle is limited by the size of cargo it can deliver, as its capacity cannot exceed 5 kb [57]. To mitigate this, studies have attempted to split CRISPR components into multiple AAVs or use smaller endonucleases such as SaCas9. Despite the simplicity of these solutions to use multiple vehicles or smaller endonucleases, multiple delivery vehicles inherently have lower targeting efficacy and higher viral toxicity and SaCas9 has its own limitations due to a less common PAM site, limiting its potential [29,56,57]. Adenoviral vectors have a large packaging capacity and do not integrate into the host genome, thereby reducing insertional mutagenesis. Despite this, they are highly immunogenic. Many studies have made use of baculoviruses, which are notably characterized by their large packaging capacity of 38 kb [58].

Despite some of the success seen in clinical trials using these viral vectors, there are many safety concerns associated with the immunogenicity of these delivery vehicles. There are also concerns over the development of antibodies against the virus, known as anti–Cas9 responses, limiting their use to one time only. Nonetheless, novel variants, such as capsid variants, are being engineered to reduce the immune response [59].

3.3.3. Non-viral delivery: To mitigate the challenges of viral delivery, researchers have explored non-viral delivery methods, which can be divided into chemical and physical methods.

3.3.3.1. Chemical methods. Non-viral chemical methods for CRISPR delivery include polymeric and lipid nanoparticle systems. These methods induce transient nuclease expression, thus reducing off-target edits and other toxicity-related responses. This is especially important for CRISPR, as opposed to traditional gene therapy, as stable

integration into the host genome is not necessary to maintain adequate therapeutic levels and would have adverse effects. Cationic liposomes and polymers are commonly used as *in vivo* delivery vehicles of CRISPR components due to their ability to electrostatically encapsulate anionic CRISPR pDNA, mRNA, or RNP complex. Researchers can create large libraries to screen for the most optimal nanoparticle formulation that has the highest packaging capacity, lowest immunogenicity, and highest editing efficiency. For instance, researchers have experimented with the branching and functional groups of the cationic polymers to enhance electrostatic interactions and increase cell uptake and endosomal escape [60]. Typically, similar polymers and nanoparticle formulations used for traditional gene delivery can be used for CRISPR delivery. Moreover, other researchers have explored using magnetic nanoparticles and nanodiamonds as a more novel approach to non-viral delivery [61,62].

3.3.3.2. Physical methods. Physical methods for the delivery of therapeutics include microinjection, electroporation, and sonoporation. These methods are characterized by being highly precise yet invasive. For instance, microinjection, the direct injection of CRISPR components into cells, and electroporation, using electric impulses to transiently open the plasma membrane, have shown to have high transfection efficacy, yet cannot be used *in vivo* for their cytotoxicity. Moreover, manipulating the cell membrane compromises the integrity of the cells [56,63]. Nonetheless, electroporation is still often used for *ex vivo* studies when cells are transfused back to the patient after they have been edited. More novel physical delivery methods include transmembrane internalization assisted by membrane filtration (TRIAMF) and induced transduction by Osmo cytosis and propane betaine (iTOP) [63,64]. These methods are characterized by being less cytotoxic than electroporation, yet they still cannot be used for in vivo delivery [63,64].

These physical methods are generally preferred for cells that have low endocytic uptake and thus rely on direct methods for transfection. Nonetheless, it's critical to keep in mind that not all clinical applications can rely on *ex vivo* editing or have access to advanced equipment.

3.4. Gene therapy and regenerative medicine

In this review, we will focus on the application of gene editing to regenerative medicine and tissue engineering. Gene editing is a specialized subset of the broader field of gene therapy, offering more precise and long-lasting alterations to the genome. Unlike traditional gene therapy, which typically involves the delivery of an exogenous gene to target cells and often requires multiple rounds of treatment to maintain therapeutic effects, gene editing directly modifies the DNA sequence within the genome [65]. Gene therapy, or gene addition therapies, presents itself in two different forms: *in vivo* delivery of an exogenous gene or *ex vivo* delivery of the gene to harvested cells followed by cell transplantation. Apart from direct genetic modifications via gene editing, gene therapy either silences the production of an overproduced protein or delivers genetic material to artificially increase the production of a protein [66]. For instance, for bone regeneration, many studies have relied on delivering angiogenic factors, bone morphogenic proteins, or osteogenic transcription factors to pluripotent stem cells to induce differentiation [67,68]. Similarly, for treating sickle cell disease (SCD), the FDA recently approved bluebird bio's *ex vivo* gene therapy which adds

a functional β-globin gene to patient cells [69]. While gene therapy has achieved significant success in treating many diseases and is considered a major milestone in the field, it falls short of providing a cure for certain conditions because it only delivers a gene without correcting or inactivating mutant genes [70]. One of the greatest challenges of traditional gene therapy is the optimization of the delivery vehicle, and we will explore throughout this review how this remains a challenge for gene-editing technologies as well. Overall, while the delivery of healthy genes to a cell or tissue can have promising therapeutic outcomes, the ability to correct a mutated gene holds even greater promise.

4. Therapeutic applications of gene editing for regenerative medicine

In this section, we will explore the therapeutic applications of gene editing for regenerative medicine. Broadly, we have divided this into four categories: correcting monogenic diseases, augmenting tissue repair, mitigating a post-transplantation immune response, and precise protein dosage and delivery.

Excitingly, many clinical trials are currently underway using CRISPR/Cas9 to treat various diseases, disorders, and syndromes. Based on data from clinicaltrials.gov, most trials in phase I/II/III aim to treat hemoglobinopathies such as SCD and β-thalassemia or various forms of cancer including lymphomas, leukemia, and solid tumors. There also exists one active clinical phase I trial underway for treating Duchenne muscular dystrophy (NCT05514249). These trials pave the way for future advancements in gene-editing therapies. Importantly, the lack of clinical trials exploring gene editing for developing tissue constructs to treat degenerated tissue highlights a significant gap in translating gene-edited therapies for regenerative medicine applications.

4.1. Gene editing to correct genetic diseases

Monogenic diseases such as cystic fibrosis (CF), SCD, and osteogenesis imperfecta (OI) are excellent candidates for treatment using genome editing technology given that their mutations are localized to a single gene. More than 5000 monogenic diseases exist, and it is estimated that they affect 6% of the population during their lifespan [71]. Gene editing technology can be applied to stem and progenitor cells to have long-lasting and potentially curative effects. The next sections discuss how researchers have leveraged CRISPR technologies to compensate for genetic diseases. We chose to focus on these three monogenic diseases due to their prevalence, extensive investigations in the field, and the promising potential for cures based on recent progress.

4.1.1. Cystic fibrosis: CF is a monogenic disease caused by mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene which leads to dysfunctional chloride channels in the respiratory and digestive tracts. Small molecule drugs have demonstrated the potential to improve lung function, yet they are limited by their demand for repeated dosing, are inapplicable in CF patients who are incapable of producing the *CFTR* gene, and ultimately fail to target the genetic root of the disease. Moreover, various extracellular barriers such as airway mucus, mucociliary clearance, and CF mucopurulent sputum, pose challenges for both drug and gene delivery *in vivo* [72]. This is especially important given the surge of gene therapy approaches that seek to deliver complementary

DNA (cDNA) for the *CFTR* gene [73]. Furthermore, achieving permanent wild-type *CFTR* expression using gene therapy is limited by the rapid turnover of pulmonary epithelial cells as well as stem and progenitor cell targetability [74]. Genetic editing by CRISPR/Cas9 bypasses this limitation given that it does not require continuous expression of the nuclease to be efficacious. Nonetheless, it's important to note that there exist over 2000 CF mutations which can be categorized into six different classes, emphasizing this disease's difficulty in targeting regardless of whether CRISPR or small molecule drugs are used [75,76].

Many studies have used CRISPR HDR-mediated knock-in to create a genetically corrected autologous stem cell therapy. In one study, researchers employ *ex vivo* gene editing to correct the commonly seen class II ΔF508 mutation in primary upper-airway basal stem cells (UABCs) [77,78]. Researchers electroporated UABCs with RNPs with silent mutations to prevent Cas9 recutting [77]. In addition, researchers delivered the donor repair template via AAV6 at a multiplicity of infection (MOI) of 10^6. Similar strategies using electroporated RNP followed by AAV6 donor delivery have successfully introduced large transgenes to hSPCs, except these cells did not require as high of a MOI [79]. Overall, results showed high correction efficiency in UABCs without using any drug-based selection methods commonly required in HDR methods. Despite the technical challenges of harvesting autologous UABCs, using these primary cells offers advantages in that it limits the likelihood of immune rejection, reduces the risk of teratoma formation caused by undifferentiated iPSCs, and can be directly transplanted back into the patient without the need for lengthy differentiation protocols (Fig. 3(a)). However, future work is needed to optimize *in vivo* transplantation protocols to the upper airway [77]. While electroporation was applicable for this study, it cannot be used for *in vivo* studies. The same research team later expanded on their work, realizing that there are many other pathogenic mutations within the *CFTR* gene to correct, in addition to the common ΔF508. Thus, they inserted *CFTR* cDNA into the *CFTR* locus using CRISPR/Cas9 HDR-mediated knock-in, where the exogenous gene was carried in two AAVs into UABCs [80]. HDR requires an exogenous repair template and is cell cycle-dependent, indicating the process is highly inefficient and infrequent for translational and clinical purposes [81]. Alternatives or improvements to traditional HDR would benefit CF applications.

An alternative approach for rectifying mutations in the *CFTR* gene involves delivering AsCas12a along with a single crRNA to correct point mutations in primary airway epithelial cells using indels. Maule et al. [74] used this approach to correct mutations in the *CFTR* gene that disrupt proper gene splicing, leading to the production of truncated and dysfunctional protein products. Other studies performed a similar tactic, leveraging SpCas9 and multiple sgRNAs to correct splicing mutations [82]. However, the deletions performed using AsCas12a were smaller, pointing to the precision of this nuclease. To correct the same mutations while seeking out more efficient editing methods than the error-prone and cycle–cycle-limited NHEJ and HDR, respectively, other studies have used base editors [83] and prime editors [84]. One study used base editing to deliver optimized SpCas9-ABE7.10 RNPs to human airway epithelia, rather than stem and progenitor cells, via electroporation to restore *CFTR* channel function. The application of base editing is possible given that many *CFTR* mutations are point mutations. Despite successful editing, this tool also gave rise to undesirable bystander editing [83].

Taking everything together, there are some important considerations to note for gene editing for CF. Foremost, regarding delivery, a CF therapeutic would benefit most from a systemic delivery that can reach many tissues, since CF affects many organ systems. This however requires passing through the capillary endothelial layer as well as the interstitial tissue to reach the epithelial basement membrane within the lung or other organs [75]. On the contrary, delivery to the epithelial lumen requires bypassing the thick mucus layer and mucociliary clearance (Fig. 3(b)) [75]. Cell-penetrating peptides (CPPs) combined with shuttle peptides can translocate across barriers and reach epithelial barrier cells [85]. Nonetheless, researchers may opt for an invasive autologous or allogeneic transplantation of corrected cells, even though this does not solve the issue of widespread diseased organ systems, it is thought that just targeting the lungs could lead to significant improvements [81]. Questions remain regarding how many cells need to be transplanted to reach therapeutic efficacy, but previous studies suggest that when 10%–50% of airway epithelial cells express wild-type *CFTR*, the system displays wild-type function [86]. Moreover, given the vast number of CF mutations, one advantageous option is to insert a full-length *CFTR* gene to replace the entire mutated endogenous gene, despite the limitations of HDR. These limitations may be overcome through prime editing for CF.

4.1.2. Sickle cell disease: Transfusion-dependent β-thalassemia (TBT) and SCD are two common monogenic diseases caused by mutations in the *HBB* gene, with both impairing red blood cell function and the latter leading to mutations in the wild-type form of hemoglobin (HbA) to sickled hemoglobin (HbS) [87]. While current therapies help manage disease symptoms, none of them have curative effects [88]. For instance, the standard-of-care, hydroxyurea, is a fetal hemoglobin-boosting agent. Fetal hemoglobin (HbF) is typically silenced after birth, but an increase in its production can compensate for disease-driven HbA deficiencies. While this oral drug has high patient compliance, this comes at the expense of many unwanted side effects and the need for repeated administration [89]. Gene therapy approaches rely on lentiviruses to introduce an anti-sickling β-globin variant or short hairpin RNA (shRNA) to reverse the repression of HbF [88,90]. The outcome produces anti-sickling hemoglobin variants (HbA^{T87Q}) or induces HbF formation, respectively [88,90].

Excitingly, the FDA approved the first one-time non-viral CRISPR/Cas9 therapy, CASGEVY (exagamglogene autotemcel), for SCD and β-thalassemia made by Vertex and CRISPR Therapeutics [91,92]. The treatment is an autologous *ex vivo* gene therapy that harvests the patient's human pluripotent stem cells (hPSCs) from the bone marrow (BM) and edits them (Fig. 3(a)) [91]. This therapeutic silences the *Bcl11a* gene, a gene that serves to repress the fetal hemoglobin gene [91]. Finally, the patient receives BM ablation therapy to remove the deficient blood cells, and the edited cells are infused back into the patient's BM. Other clinical trials aim to treat the disease in this same manner by increasing the amount of HbF (NCT06506461). Given the invasiveness of harvesting patient cells, myeloablation, and transplantation, more research is needed to evaluate the technology's potential when delivered *in vivo*. The financial expense of this therapy for SCD and β-thalassemia remains a barrier, especially given that the disease is prevalent in many resource-poor regions. Moreover, re-activating HbF still leaves the root cause of the disease

unfixed. Nonetheless, by precisely editing stem cells, this therapy has the potential to be curative.

Given that SCD is caused by a single point mutation in the *HBB* gene, studies have attempted alternative strategies for correction. One autologous drug product, nulabeglogene autogedtemcel (nula-cel) by Kamau Therapeutics, is in phase I/II and relies on HDR to convert the mutant amino acid thymidine to the non-pathogenic variant (NCT04819841). In the one-year follow-up on the first patient treated with nula-cel, the patient showed significant clinical improvement with no vaso-occlusive crises reported and improved quality of life, supporting the drug's potential to treat additional patients [93]. The mechanism of action of nulacel is distinct from CASGEVY, as it targets the disease at its root by reducing the level of pathogenic HbS. While the long-term durability of its curative impact is still being evaluated, this therapeutic represents a significant milestone in the treatment of SCD.

Considering the low efficiency of HDR and the advancement of more sophisticated base editing techniques, recent studies have focused on using prime editors to treat SCD. Li et al. [94] successfully converted the mutated valine codon to a glutamic acid codon using prime editors. Given the large size of prime editors and the size limitations of most lentiviral and AAV vectors, the cargo was inserted into helper-dependent adenoviral (HDAd) vectors which can specifically target hematopoietic stem cells (HSCs) through their fiber protein. The conversion to glutamic acid, both corrects the sickling mutation and halts the prime editor from further editing by introducing a silent mutation that disrupts the PAM sequence [94]. The *ex vivo* editing results were validated by next generation sequencing (NGS) and confirmed a high conversion rate at the target site. While *ex vivo* approaches allow a high level of controlled processing of stem cells and allow edited cells to be validated before transplantation, this time-consuming and invasive process would still benefit from *in vivo* editing. To evaluate the prime-editors *in vivo* potential, researchers intravenously injected a nonintegrating, prime editor-expressing viral vector which ultimately resulted in 43% of sickled hemoglobin being replaced by healthy adult hemoglobin [94]. *In vivo*, HSCs are found within the BM and are protected by physical barriers, such as the BM stroma. Currently, *in vivo* editing strategies rely on mobilizing the HSCs from the BM to the peripheral bloodstream, to be accessible to intravenously delivered gene-editing components (Fig. 3(b)) [94,95]. Optimizing delivery directly to the BM would benefit these strategies. Nonetheless, prime editing for SCD has made great strides in both *ex vivo* and *in vivo* approaches, with both methods exhibiting normal hematological parameters, similar to healthy controls [94]. This therapeutic offers minimal off-target editing, simplicity, and portability allowing it to be used in underdeveloped regions where the disease is widespread.

4.1.3. Osteogenesis imperfecta: OI is a monogenic bone disease characterized by brittle bones, decreased bone mass, and repeated fractures. It is known to be associated with mutations in the *Col1a1* and *Col1a2* genes, which translate to protein products that make up type I collagen, a main constituent of bone. Apart from bisphosphonates to prevent bone loss, researchers have attempted stem cell therapy. Researchers transplanted human fetal blood mesenchymal stem/stromal cells or human fetal early chorionic stem cells either intraperitoneally or into bones of OI mice, which differentiated into mature osteoblasts,

ultimately producing healthy collagen [96,97]. Despite this success, it fails to tackle the disease at its genetic origin.

In one study, researchers used OI patient-derived peripheral blood mononuclear cells (PBMCs), reprogrammed to iPSCs, and corrected the mutated gene using HDR-mediated CRISPR knock-in [98]. For this *in vitro* experiment, the endonuclease and the donor single-stranded DNA (ssDNA) were transfected using a reagent, and the cells successfully differentiated into osteoblasts producing type I collagen [98]. In another study, researchers similarly used OI patient-derived fibroblasts and simultaneously reprogrammed these cells into iPSCs while also delivering CRISPR/Cas9 components along with donor DNA for HDR-mediated mutation correction [99].

These studies serve as proof-of-principle for OI gene correction, but future work is needed to assess the ability to transplant these cells into the patient. Evading a post-transplantation immune response is a critical feature of success and will be discussed in Section 4.3.

4.1.4. Additional disease correction applications: There have been many other diseases that CRISPR technologies have successfully been able to correct, such as Duchenne muscular dystrophy, diabetes, and age-related macular degeneration (AMD). Here we have briefly listed studies and their successes and limitations.

To combat the challenging delivery of large ABEs, researchers have split the base editor into two separate viral vectors. Using dual AAVs, researchers were able to correct Duchenne muscular dystrophy in adult mice [100]. Despite this success, positive results in mice do not necessarily translate to humans, as a phase I clinical trial for treating Duchenne muscular dystrophy resulted in the death of the patient, due to the high dose of intravenously delivered AAV, encoding CRISPRa editing technologies (NCT05514249). This reflects that though *in vivo* approaches are more ideal for delivering CRISPR/Cas9 than *ex vivo* approaches, the field has yet to optimize the *in vivo* delivery and efficiency, sufficiently to decrease the dosage while maintaining a high level of gene correction and targeting. Additionally, there is a requirement for delivery systems that are less toxic than viral vectors, as viruses can be highly toxic, especially when administered in high doses, which is often necessary for delivering larger CRISPR technologies.

In another study, researchers corrected a diabetes-causing pathogenic variant *ex vivo* using CRISPR HDR [101]. For AMD treatment, researchers used engineered lentiviruses to create transient Cas9 expression for CRISPR knockout of the target gene, effectively restoring retinal tissue and blood vessel development while also mitigating one of the main challenges of lentiviral delivery—integration into the host genome [102]. Apart from optimizing *in vivo* delivery, efficiency, and scalability, future work will explore creating off-the-shelf corrected iPSCs to ease *ex vivo* interventions. This is challenged by the fact that each patient's immune system is variable and the same disease may have different clinical presentations.

4.2. Gene editing for augmenting tissue repair

Many diseases and injuries are characterized by a loss of mature cells. For example, neurodegenerative diseases such as Alzheimer's disease, amyotrophic lateral sclerosis,

Parkinson's, and Huntington's disease are accompanied by both loss and degeneration of central neurons. Moreover, bone and muscle injuries are characterized by a loss of osteoblasts, chondrocytes, as well as muscle fibers. These cells are often permanently lost or struggle to regain complete function and quantity. Also, the regenerative potential of many of these cells is lost with age. Thus, the field of tissue engineering relies heavily on iPSCs and ESCs which have advanced pluripotency and self-renewal abilities, as well as high differentiation potentials for tissue replacement therapies. Limits in the availability of human embryos as well as ethical concerns have driven the field to seek methods to optimize the creation of iPSCs. Lentiviral delivery of transcription factors for reprogramming somatic cells has dominated the field for many years, despite its limitations in targeting and multiplexing, as well as its incompatibilities with large transgene sizes. Moreover, after the iPSCs have been dedifferentiated, achieving directed *in vitro* differentiation using carefully selected culture media to obtain highly specific and purified cell populations is challenging and oftentimes lacks reproducibility.

In this section, we will review applications of CRISPR technologies in the general space of augmenting tissue repair. This begins with using CRISPR technologies to drive somatic cell reprogramming into iPSCs. Next, we will explore considerations when using CRISPR technologies to differentiate mature cell types. Finally, we discuss the transplantation of these cells to create *in vivo* tissue constructs to repair damaged tissue. Together, all these applications use CRISPR technologies to redirect cell fate for regenerative purposes. Fig. 4(a) summarizes these applications.

4.2.1. Reprogramming somatic cells to iPSCs: Reprogramming of mature, somatic cells into pluripotent cells has historically been done by introducing a well-studied set of exogenous transcription factors characterized by their importance in embryonic development: *Oct14*, *Klf4*, *Sox2*, and *c-Myc* into the respective open reading frame (ORF) [103]. Future studies have seen success using additional transcription factors such as *Nanog* and *Lin28* [104]. These have been delivered via plasmid viral vector expression, mRNA, or protein transfection. However, these methods have their limitations: plasmids and viral vectors result in unwanted integration into the genome fostering reactivated exogenous gene expression potentially leading to tumorigenesis [105], and retroviral vectors specifically have temporal and leaky exogenous gene expression as pluripotent cells silence retroviruses [106]. Moreover, non-integrating viral vectors, such as adenoviruses, have been demonstrated to have limited reprogramming efficiency, and protocols for using synthetic mRNAs and proteins are both costly and technically challenging [107,108]. Recently, studies have relied on chemical methods for somatic cell reprogramming, which poses its own challenges in efficiency, specificity, and chemical-induced genotoxicity [109,110]. To combat these limitations, we will explore the use of CRISPR technologies for somatic cell reprogramming.

CRISPRa/CRISPRi offers advantages specifically for genetic reprogramming. The technology has simultaneous multiplexing capability, allowing diverse sgRNAs for targeting promoters at different loci to be concatenated in a single plasmid. Moreover, it has high specificity for targeting the multiple endogenous loci. This paves the way for efficient and precise modifications through CRISPRa/CRISPRi. Many studies have explored the use of

CRISPRa for reprogramming somatic cells into iPSCs by targeting endogenous promoters, such as *Oct14*, *Klf4*, *Sox2*, *Lin28*, and *c-Myc* [111]. In addition, studies have found that reprogramming efficiency can be further improved by additional targeting of the embryo gene activation enriched ALU motif (EEU), which is enriched near promoters of genes involved in embryo gene activation (EGA), as well as the miR-302/367 locus, which is expressed at high levels in ESCs [111,112]. These methods have also been demonstrated to increase the kinetics of iPSC formation compared to conventional methods [111,112]. The specificity and longevity of gene editing for reprogramming somatic cells make it stand out among viral delivery of transcription factors.

4.2.2. Considerations when differentiating iPSCs to desired cell types:

Collectively, the field has been able to differentiate stem and progenitor cells into neurons, monocytes, macrophages, skeletal muscle, osteoblasts, and chondrocytes [42,44,113–115]. The success of these studies is attributed to a carefully chosen activator or repressor domain which is fused to dCas9. For instance, the VPR activator was shown to promote neuronal cell differentiation at least ten times higher than VP64 [44]. However, there exist other crucial factors contributing to successful transcriptional regulation, including the epigenetic state of the target gene, the size of the CRISPR components, and the capacity to finely control dCas9 activity.

Some studies have focused on direct transdifferentiation from one mature cell type to another. In one study, researchers converted astrocytes to functional neurons *in vivo* by activating three endogenous genes using a SunTag transcriptional activator with p65-HSF1 replacing VP64 [26]. As with other dCas9 systems, the researchers found that the target genes were activated at different levels which they hypothesize is due to basal expression levels, epigenetic status, or accessibility of sgRNA target sites [26]. Thus, this data highlights the importance of extrinsic factors affecting the success of CRISPR technologies and the subsequent protein expression. Efficacy depends not only on the efficiency of the transcriptional activator but also on the epigenetic status of the target gene and the sgRNA's ability to localize it.

Moreover, *in vivo*, delivery of large dCas9 and transcriptional activators/repressors remains a challenge that limits the full translational potential of this technology. This is significant given that the fused domains and regulators are large and often used in combination to increase efficiency. The size of dCas9 with transcriptional domains is larger than the capacity of recombinant AAV vectors, the gold standard for gene delivery. To mitigate the dCas9 delivery challenges, studies have attempted to split the dCas9 at the protein level into two delivery vehicles which has successfully shown efficient transcriptional activation and long-term expression of the target gene in some studies [116]. The reconstitution efficiency of the two halves varied greatly depending on the site of the splitting. Thus, these studies would benefit from an in-depth screening and characterization of the effect of splitting sites on reconstitution efficiency, and to what extent this affects transcriptional activity. Another study split the CRISPR components by expressing the sgRNAs and transcriptional activators/repressors in one virus and the dCas9 in another [117]. Despite the success of dual viral delivery of dCas9, the safety concerns associated with one, or multiple, viral vectors

remain an issue. Moreover, these dual delivery systems typically have lower efficiency due to difficulties in temporally and spatially delivering both components into a given cell [118].

Thus, successful non-viral delivery of dCas9 and sgRNA through chemical or physical methods would presumably have a higher loading capacity, thus presenting a more efficient and safe option for large dCas9 delivery. In one study, researchers explored a self-assembled peptide (SAP) coating on polycaprolactone (PCL) nanofibers forming SAP-coated scaffolds that can successfully load and release CRISPR components through the help of a polyethyleneimine (PEI) based transfection reagent (Fig. 4(b)) [119]. The combination of PCL which is wildly used in tissue engineering for its structural stability and biocompatibility with coated SAP for localized CRISPR delivery allows for customizable scaffolds, that can incorporate unique and functional peptides. These peptides can serve different purposes, such as proliferation or cell adhesion, complementing the transcriptional activity by CRISPR. For instance, laminin-derived peptides can be conjugated to facilitate neuronal adhesion concurrent to CRISPRa-mediated neuronal differentiation. Despite the exhibited success of loading and release of pDNA complexes, the transfection efficacy was not high, especially in hard-to-transfect cell lines, such as human neuronal progenitor cells (hNPCs) [119]. The authors suggest that this may be improved via the delivery of RNP components, despite its expense, or a more effective delivery vehicle than the PEI transfection reagent. Thus, this still demands the necessity of a non-viral CRISPR delivery system with high transfection efficiency. In another attempt to achieve high-capacity delivery, studies have explored using smaller variants of dCas9 by deleting various domains. Despite this, these methods are not flawless, as they pose limits on the available PAM sites that can be used. Studies have also explored using smaller-sized techniques for epigenetic control, other than CRISPR technologies, such as nanobodies to recruit endogenous chromatin regulators to a target gene and ultimately create epigenetic memory [118]. Even though gene-editing techniques, specifically CRISPR technologies, are the focus of this review, it is worth noting that other, smaller, alternatives exist for transcriptional regulation. Combination therapies utilizing both techniques could create stronger transcriptional regulation.

Moreover, successful directed differentiation is reliant on methods that increase control over dCas9. This creates more efficient regulation of gene activation or inhibition, especially given the fact that most applications require the constitutive and maintained expression of dCas9 for transcriptional control throughout many cell divisions [42]. Multiple studies found that simultaneous targeting of the same gene using multiple sgRNAs produced an increase in the desired transcriptional activation [47,120,121]. In another system, researchers combined dCas9 with KRAB and DNA methyltransferases along with proteolysis-resistant linkers to stabilize gene-silencing memory [42]. The study found that despite transient dCas9 protein expression (ten days post-transfection), transcriptional activity is sustained, remaining for 50 days, which proved to be helpful in their application in silencing endogenous genes for iPSC-derived neuronal differentiation [42]. While some therapeutic applications may benefit from sustained gene expression as the cell progresses such as housekeeping genes, required for cell survival and maintenance, other applications may only require transient gene expression such as genes involved in a cell’s stress response. In this study, researchers found that they can efficiently reverse the previous transcriptional

silencing via DNA methyltransferase knockout, also using CRISPR [42]. Together, these studies suggest programmable strategies towards epigenetic memory during cell division and differentiation.

Regarding somatic cell reprogramming, as discussed in Section 4.2.1, researchers have delivered doxycycline (DOX)-inducible transgenes given that reprogramming efficiency is correlated with the duration of transgene expression [26,122]. Although DOX-induced technology has historically been applied to lentiviral transcription factor delivery for reprogramming, recent studies have utilized DOX-induced reprogramming in conjunction with CRISPRa/CRISPRi technologies to eliminate unwanted reactivation and inefficient silencing. dCas9 is integrated into the host genome, and DOX is used to control pluripotency and differentiation, ultimately increasing or decreasing protein levels [116,123,124]. Thus, we can now activate certain genes, but with much greater control, such as only when the cell has reached the appropriate state of maturity. Moreover, DOX-induced Cas9 expression resolves the issue of leaky Cas9 expression, which contributes to unwanted and off-target editing. This tunable and reversible gene activation technology is particularly useful, given that cell states are transient and there is a temporal correlation for activating transcription factors during mature cell differentiation.

In one example, researchers used CRISPR knock-in to integrate exogenous transcription factors known to be essential for the establishment of motor neuron (MN) identity downstream of the inducible tetracycline response element [125]. Despite the integration of these transgenes, the authors observed a dysregulation in endogenous regulatory and functional genes that promote MN differentiation [125]. This was seen in conjunction with leaky expression using tetracycline-inducible systems as well as the transcription factors becoming activated even before DOX was introduced. Overall, differentiation failure was accredited to the premature activation of the transgenes in iPSCs, which disrupted the proper neuronal differentiation process [125]. This study sheds light on the drawbacks of DOX-inducible systems as well as the challenges at play when using CRISPR technologies to drive differentiation without using patterning factors that would have been supplied in media. Not to mention another drawback of these systems when the drug's threshold level is reached, there is an inverse correlation between drug concentration and gene activation/repression [116].

In summary, directed differentiation of iPSCs into mature cells is crucial, considering the numerous degenerative diseases and injuries marked by cell loss. While applying CRISPR technologies to iPSCs presents a valuable option, it's essential to account for factors such as the epigenetic accessibility of the target gene, the size of editing components, and attaining programmable nuclease expression, as highlighted by the studies.

4.2.3. *Tissue constructs* in vivo: While traditional autografts and allografts exist that don't rely on gene-editing technologies, these methods are limited by their availability, low efficiency, and the chance of infection. Coupling gene-editing technologies with stem cells that possess high proliferation and differentiation capacity heightens the regenerative potential of the cells and can pave the way for complete regeneration. In this section, we will review applications of CRISPR technologies to stem cells to repair damaged tissue *in vivo*.

4.2.3.1. Bone repair. While the skeleton is capable of regenerating itself, bone regeneration is limited in cases of critical-sized bone defects and large bone losses [126]. Furthermore, while infants can repair calvaria bone easily, adults lose this capability with compromised stem cells that come with age [127]. Also, in the case of bone tumors, which are common in cancer metastasis, the bone cannot regenerate on its own, rendering the necessity of novel bone repair techniques [128]. Many studies we reviewed here have leveraged genome-edited cells, rather than gene-delivered cells, to graft back into patients to repair damaged bone.

Studies have shown that growth factors such as bone morphogenetic proteins (*Bmp*), insulin-like growth factors 1 and 2 (*Igf1* and *Igf2*), transforming growth factors (*Tgf*), and vascular endothelial growth factor (*Vegf*) can lead to osteogenesis and are present during the natural bone regeneration process [67,68,129]. As such, researchers have explored activating these genes using CRISPRa to regenerate bone tissue. In one study, researchers edited primary BM mesenchymal stem cells (MSCs) *ex vivo* using lentiviral CRISPRa to overexpress *Bmp9*, and subsequently locally injected the cells into rats with calvaria defects [130]. The study used constitutive dCas9-VPR expression. *Bmp9* increased the osteogenic potential of MSC differentiation, and *in vivo* studies demonstrated an increase in bone formation and mineralization [130]. Despite lentiviral transfection efficiency, its size limitations and safety concerns remain a challenge. In another example, researchers co-activated *Wnt10b* and *Foxc2* genes using a baculoviral SAM-based CRISPRa system in primary BM stem cells to promote osteoblast differentiation and subsequently implanted the cells *in vivo* for bone repair in calvaria bone defects [121]. Here, the CRISPRa system successfully activated the endogenous genes for longer than 14 days [121]. The baculovirus is characterized by its high packaging capacity compared to lentiviruses allowing it to fit all necessary CRISPRa/CRISPRi components. Moreover, it has non-integrative properties, which reduce toxicity and support it as safe, but limit the duration of activation. To mitigate limits on the duration and prolong the expression of the activated gene, the researchers here used a hybrid baculovirus system expressing Cre recombinase and a separate loxP flanked transgene composed of the sgRNA sequence, dCas9 and the associated transcriptional activator domains, and nuclear localization signals [121,131]. In another study, researchers co-activated transforming growth factor beta-1 (*Tgfb1*) and *Vegfa* using non-virally delivered CRISPRa to promote osteogenesis of pre-osteoblast cells (Fig. 4(c)) [132]. The researchers used a cationic copolymer with nucleus-localizing peptides and groups with tertiary amines. This non-viral delivery system mitigates the packaging limitations and immunogenicity concerns of viral vectors and includes measures to effectively deliver CRISPR components to the nucleus to bypass plasmid lysosomal degradation [132]. Moreover, to offset the risks associated with invasively administering edited cells as was done in other studies, here researchers implanted edited cells in a hydrogel for *in vivo* delivery. Thus, this study highlights not only the need for adequately edited cells but also for a cell-encapsulated transplantation device that mimics the natural ECM and offers tunable cell release. Here, the multiplexed gene activation had greater improvements in bone density and healing than the single gene activations, showcasing the advantage of the multiplexing potential of CRISPR technologies (Fig. 4(c)). Nonetheless, this non-viral CRISPRa delivery system, is limited by the duration of its activation effects, as it did not surpass 14 days, which

is hypothesized to be due to enzyme DNA degradation. This may not be suitable for long-term bone regeneration processes which require sustained gene activation for tissue repair. This suggests the need for an inducible or programmable non-viral CRISPRa system for controlled and sustained growth factor activation yet still with a high safety profile. Previous work has relied on biodegradable and biocompatible scaffolds to directly load growth factors, such as BMP9, which effectively promotes the differentiation of MSCs into osteoblasts [133]. Though direct gene therapy and protein delivery seem to be plausible, they are limited by their transient expression and inefficiency in targeting, and if inserted into a scaffold, bioactivity losses [134]. The precision offered by CRISPR/Cas9 as well as its multiplexing potential makes this option stand out among traditional gene or protein delivery. However, it is critical to assess when multiplexing capabilities will be advantageous, as some growth factors are known to act independently, while others function best synergistically [68]. It's also important to note that many of the genes mentioned such as *Tgfb1* and *Foxc2* have pleiotropic roles. Caution should be used when activating these molecules and it is advised to use non-integrating delivery vehicles to help create temporal control over transgene expression.

In addition to CRISPRa, CRISPRi can also effectively promote bone regeneration. While other methods of protein repression exist such as siRNA and shRNA, these methods are prone to off-target effects. CRISPRi has been demonstrated to have the fewest off-target effects when compared to RNA interference (RNAi) and antisense oligonucleotides [135,136]. *Bmp2*, while promoting bone repair, also promotes the expression of Noggin (*Nog*), an antagonist that self-restricts its activity [137]. Thus, researchers have concurrently inhibited *Nog* and introduced *Bmp2* in adipose-derived stem cells (ASCs) using a hybrid baculovirus to stimulate effective osteogenesis and *in vivo* bone healing [131,137]. Further, while this study did not use CRISPRa for *Bmp2* activation, future studies could seek to express both CRISPRi and CRISPRa in a single baculovirus given its large size. For instance, in another study, researchers created a bidirectional gene regulation system capable of both activating and repressing genes to induce chondrocyte differentiation to promote bone healing [138]. Specifically, researchers activated *Sox5*, *Sox6*, and *Sox9* using nuclease-dead *Streptococcus pyogenes* Cas9 (dSpCas9) fused transcription activators and chromatin remodeling methods, namely histone acetylation, and repressed *C/ebp-α* and *Ppar-γ* using DNA methylators fused to nuclease-dead *Staphylococcus aureus* Cas9 (dSaCas9)_ in ASCs [138]. These techniques exploit ASCs, rather than BM stem cells, which are easier to acquire but also are less prone to osteogenesis or chondrogenesis, thus necessitating the use of CRISPR/Cas9. In this system, researchers delivered the CRISPR components using three baculoviruses given the large number of genes targeted [138].

In both CRISPRa and CRISPRi systems, activation or repression efficiency depends on the local chromatin structure and accessibility which directly implicates the ability of dCas9 to reach the endogenous target gene. This suggests an important consideration when determining which gene to target [139]. Moreover, in all of these studies, it is critical to screen the effect of different or additional transcriptional activators or repressors that are fused with dCas9 as well as the success of using alternate cleavage systems to CRISPRi such as Cas13a, Cas13b, and Cas13d [140,141]. Finally, as mentioned in some studies, in addition to transcriptional activators and repressors, it is beneficial to include epigenetic

and chromatin remodeling agents, which is especially advantageous for hard-to-target genes [138].

4.2.3.2. Other regenerative and restorative applications. In addition to transplanting edited stem cells to patients requiring bone repair, this technology has been used for reconstructing many other damaged and degenerated tissues.

For the treatment of corneal endothelial diseases, which suffer since wild-type human corneal endothelial cells (hCECs) do not regenerate, researchers have applied CRISPRa to reverse degenerative characteristics [142]. Researchers activated *Sox2* which ultimately promoted wound healing and regeneration of hCECs [142]. Here, the researchers used a local injection of plasmid into the anterior chamber coupled with electroporation, two days before corneal injury [142]. Injecting edited cells before injury is unseen in many gene-engineered regenerative medicine applications but should continue to be studied in other diseases for its prophylactic potential.

Moreover, developers of the hybrid baculovirus system previously mentioned used this technology for activating three neurotrophic factor genes (*Bdnf*, *Gdnf*, and *Ngf*) in ASCs using SAM-based CRISPRa [143]. When delivered to sciatic nerve injury sites *in vivo*, animals exhibited functional recovery, nerve reinnervation, axon regeneration, and remyelination [143]. Moreover, the system enabled lengthy therapeutic expression, with genes activated for at least 21 days, accredited to the Cre/loxP system unique to the hybrid baculovirus [143]. Despite the success of this study, delivery of cDNA encoding for one neurotrophic factor had higher expression than that of the CRISPRa system. Nonetheless, CRISPRa is still programmable and capable of multiplexing, which traditional gene therapy lacks [143].

CRISPR technologies have extended beyond editing stem and progenitor cells to include mature cells for addressing type II diabetes and obesity-related diseases [144]. These edited cells, upon transplantation, exhibited vascularization, a crucial aspect of tissue engineering and *in vivo* construction. The inclusion of edited stem and progenitor cells in this study could enhance its potential to deliver lasting and potentially curative effects.

4.3. Gene editing to prevent post-transplantation immune response

The full translational potential of iPSCs or engineered tissue constructs is only realized when researchers consider transplantation strategies to evade immune rejection and graft-versus-host disease (GVHD). Moreover, the only way to create off-the-shelf or universally compatible therapies is to recognize and prevent immune mismatches. It is especially important to study the immunogenicity of iPSCs, as they are known to increase in their immunogenicity as the progeny differentiates [145]. Current tissue engineering strategies seek to create novel immunomodulatory biomaterials that mitigate transplantation toxicity, have long-term durability, and promote a pro-regenerative immune microenvironment [146]. On the other hand, many recent studies have relied on CRISPR knockout to evade post-transplantation rejection. We surmise that the multiplexed, patient-specific, and long-lasting modifications of CRISPR edits combined with immunomodulatory biomaterials as a scaffold to carry edited cells will have high success rates.

This subject is widely explored in its application to immunotherapy, in addition to tissue engineering and regenerative medicine. For instance, despite the success of engineered T-cells for cancer immunotherapy, grafting these cells remains challenging due to host–donor mismatches, leading to an allogeneic immune response [147]. Thus, we will adapt the immunotherapy findings for our learning. In the regenerative medicine space, CRISPR Therapeutics is actively using *ex vivo* gene editing technology to protect transplanted β-cells from a patient's immune system and for diabetes treatment.

Each person's immune system is highly variable and is dependent on age, sex, history of infection or disease, inherited genetic traits, and environmental factors [148]. Thus, tissue-engineered products have variable repair outcomes in each patient. In this section, we will focus on this idea, specifically from the perspective that the immune system is an antagonist whose effects need to be evaded. Nonetheless, the immune system also has positive implications in tissue repair. For instance, B cells promote the closure of skin wounds by releasing inflammatory cytokines [149] and $CD161^{+}$ regulatory T cells accelerate wound healing [150]. To wholly realize the potential of a tissue-engineered product, CRISPR technologies can be used to both evade a harmful immune response from the graft and also to enhance the patient's host immune system to promote tissue repair, which both take into account a patient's unique immune state.

4.3.1. Human leukocyte antigen matching: An allogeneic immune response from the grafted T cells is largely due to human leukocyte antigen (HLA) incompatibilities between the donor and recipient, requiring HLA genome editing [147,151]. The same principle can be applied to regenerative medicine, specifically in the form of HLA matching or HLA editing. HLA is classified into HLA I and HLA II, which correspond to major histocompatibility complex (MHC) I and II, respectively, and present antigens to T cells, which subsequently kill the cell. Studies have shown that syncytiotrophoblast cells between maternal blood and fetal tissue during pregnancy are characterized by low MHC I and II expression and high CD47 expression. This is the likely explanation for why the maternal immune system can protect fetal cells that contain paternal-inherited antigens [152]. Ultimately, HLA profiles allow the immune system to distinguish between self and non-self [145]. Evidently, matched HLA types are necessary for successful transplantation. However, this comes at a high price, given that matching is very difficult given the genetic variation in the genotype. For instance, siblings only have a 25% chance of being fully HLA-matched [153]. Moreover, though HLA-matched hematopoietic stem cell transplantation was explored as a therapeutic option for treating SCD, it is limited by the fact that only 14% to 20% of patients have HLA-matched donors [88].

In one attempt to mitigate HLA-induced graft rejection, researchers have attempted to match HLA profiles by creating HLA-homozygous iPSC cell line banks. Thus, they used CRISPR to knockout heterozygous *HLA-B* from iPSCs with homozygous *HLA-A* and heterozygous *HLA-B*. The subsequent cell line exhibited less immunogenicity than the wild-type cell line [153]. Despite this success, results have yet to be shown demonstrating whether creating homozygous HLA profiles is enough to protect cells during allotransplantation. Moreover, other studies have demonstrated that altering the HLA profile affects a natural killer (NK) cell response, which is disregarded in many studies but important for allotransplantation

success. Moreover, this attempt to create homozygous iPSC cell line banks relies on CRISPR knockout using the traditional Cas9 nuclease which creates many different clones with variations in the indels, and ultimately only one clone produced the desired response at both the RNA and protein levels, suggesting poor efficiency [153]. Another study observed a similar phenomenon of a low success rate with its HLA knockouts as 40 clones were produced, but only three had the desired function and lacked unwanted mutations [151].

While matching HLA profiles in some studies has been demonstrated to be successful, many studies found that HLA matching was not enough to evade immune rejection. One study used iPSCs with MHC-matched profiles in macaques with and without the use of immunosuppressants for differentiation into cardiomyocytes for the treatment of end-stage heart failure [154]. MHC-matched iPSCs that underwent subcutaneous transplantation without the administration of any immunosuppressants had increased NK cell activation compared to controls with the use of immunosuppressants [154]. Another study also using matched HLA haplotypes for neuronal grafts for Huntington's disease found similar results [155]. Together, these studies demonstrate that HLA matching must be supplemented by immunosuppression to enable long-term survival and efficacy of the grafts.

To mitigate these consequences of mere HLA matching, researchers have attempted additional HLA knockouts. In one attempt, researchers knocked out *HLA-A, HLA-B*, and *HLA-II* from iPSCs (but retained *HLA-C* which is critical in preventing an NK cell response) [156,157]. This strategy not only adequately creates immunologically compatible cells but also reduces the number of iPSC clones to create comprehensive haplobanks, compatible with a greater number of recipients. However, in cases where the host is heterozygous for *HLA-C* and the donor is homozygous, which can be likely given how polymorphic *HLA-C* is, this can initiate an immune response due to a lack of inhibitory NK signals [145,156]. In another example, researchers knocked out *HLA-I* and *HLA-II* by targeting the *B2M* and *CIITA* genes, respectively, to generate iPSCs that successfully evaded T and NK cell recognition [158]. The study also included a *PVR* knockout and *HLA-E* transduction to inhibit NK cell responses, which is possible through CRISPR's multiplexing capability [158]. It's important to note that the *CIITA* gene which was knocked out in this study and many others not only knocks out *HLA-II* genes, but also other immunological genes, which has the potential to lead to many side effects [151]. Similarly, another study knocked out *MHC I* and *MHC II* and over-expressed *CD47* to generate fully functional and hypoimmunogenic iPSCs [152]. These stem cells which differentiated into endothelial cells (ECs), smooth muscle cells, and cardiomyocytes all evaded immune rejection in fully MHC-mismatched recipients and had long-term survival without using immunosuppressants [152]. A final study knocked out *HLA-A, HLA-B*, and *HLA-II* in HLA homozygous iPSCs [151]. Homozygous iPSCs were selected for their simplicity in inducing a biallelic knockout using a single sgRNA, given that using more sgRNAs introduces a greater likelihood for off-target edits and mutagenesis [151]. This study suggests one of the greatest challenges of HLA editing: designing a proper sgRNA that can target both alleles of the HLA gene, given that these genes are highly variable among people and alleles [145,151].

Regarding applications in regenerative medicine, it's important to note that off-target HLA edits have many implications, including the effect on differentiation potential. iPSCs

that had large deletions in sequences between *HLA-A* and *HLA-B* lost their ability to differentiate into cardiomyocytes, which is attributed to the loss of the *POU5F1* gene, alternatively *OCT3/4*, which has roles in somatic cell reprogramming [151]. Future work should attempt to increase the precision of the edits to mitigate these consequences.

4.3.2. Preventing natural killer cell response: It is known that modifying a cell's HLA profile elicits NK cell cytotoxicity [158]. For instance, the expression of some HLA-I markers serves as an inhibitory ligand for NK cells, allowing these killer cells to recognize the iPSC as "self" [154,158]. Thus, total silencing of *HLA-I* may suppress cytotoxic $CD8^+$ T cells, but it will activate NK cells, which then requires additional genomic modifications to counteract this effect. This phenomenon is known as the "missing-self theory" and is known to elicit immune rejection [145,152].

As an alternative to using immunosuppressants to repress the NK cell cytotoxicity, researchers have attempted alternate gene-editing strategies using proteins that have known immunosuppressive properties during pregnancy. In one study, researchers upregulated CD47 expression using lentiviral gene therapy since CD47 has high expression in syncytiotrophoblast cells implying its inherent immune-inhibitory effect and has been demonstrated to prevent NK cell IFN-γ release [152]. These knockout *MHC-I* and *MHC-II* (via *B2M* and *CIITA* knockout, respectively) and *CD47* upregulated cells demonstrated long-term survival in a humanized mouse model [152]. Alternatively, studies have expressed MHC-ligands such as *MHC-E* and *MHC-G* to inhibit NK cell cytotoxicity towards hPSCs [159,160]. One study used lentiviral gene therapy to deliver *scHLA-E* for its NK cell-inhibitory properties. However, given that *HLA-E* is only a ligand for $NKG2A^+$ NK cells, and this receptor is only a subset of 50% of NK cells in healthy individuals, the researchers also had to knockout *PVR* using CRISPR to inhibit NK cell cytotoxicity on $NKG2A^-$ NK cells [158]. Though this strategy was effective, it requires multiple genome modifications, which increases potential sources of error. Another study effectively used CRISPR to biallelically knock *HLA-G* into the *B2M* loci of hPSCs [161]. Many studies previously mentioned knock out the entire *B2M* sequence coding for all *HLA-I* genes to reduce immunogenicity [147,152,158]. However, given the function of some *HLA-I* genes in inhibiting NK cell cytotoxicity, it may be beneficial to selectively knock out *HLA-I*, as was done in the study by Kitano et al. [151].

The studies mentioned the use of human progenitor stem cells for their HLA knockouts, and found that HLA knockouts promote NK cell cytotoxicity based on the "missing-self phenomenon." Another study used CRISPR to drive HLA knockouts in committed progenitor cells that differentiated into ECs. Despite having the same *B2M* and *CIITA* knockouts, these cells did not elicit an immunogenic NK cell response, which appears contradictory to the "missing-self phenomenon" [162]. Moreover, the ECs retained their vasculogenic potential when the graft was transplanted. These results are consistent with the fact that NK cell responses to vascularized tissues and solid organ allografts require additional signals to initiate cytotoxicity. Thus, though the mechanism is poorly understood, we know that NK cells are responsible for killing a hematopoietic graft, but not a tissue graft, for grafts lacking HLA [162]. While using committed progenitors has benefits in immunogenicity as indicated by this study and they are less likely to give rise to teratomas

unlike their pluripotent stem cell counterparts, they have also lost their ability to differentiate into multiple cell types.

There have been a few examples that used CRISPR technologies to evade an immune response specifically for regenerative medicine applications. In one example of chronic limb ischemia (CLI), researchers knocked out *HLA I/II* and upregulated *CD47* in iPSCS to differentiate into engineered ECs which were then transplanted *in vivo*. The cells survived, proliferated, improved limb perfusion, and reduced the likelihood of limb complications [147]. Moreover, these engineered iPSCs were also validated in a cryoinjury-induced myocardial infarction model, with transplantation showing improvement in contractility, left ventricle stroke volume and stroke work, and cardiac output [147]. This holds promise as an allogeneic therapy, given that clinical trials using cell therapy for CLI have so far only used autologous, patient-derived cells [163].

One concern over HLA-deficient grafts is the long-term non-immunogenic risks. However, one study found that HLA-I-deficient tumors expanded γδ T cells, rather than $CD8^+$ T cells, rendering the tumors still susceptible to T cell cytotoxicity [164]. This indicates the interplay of other immune cells despite HLA deficiencies. This highlights both a strength and a weakness of HLA deficient grafts, opening new doors to cells that may be involved. Nonetheless, HLA deficiencies are still a concern and are associated with bare lymphocyte syndrome (BLS) [145].

In this section, we reviewed strategies to prevent an immune response following graft transplantation. Table 1 [147,151–153,156–158,161,162] summarizes many of the strategies, as well as the limitations of these methods. While overall these strategies were found to be successful, simply the mere act of transplantation is immunogenic by attracting neutrophils and macrophages to the site, and the studies we have presented have not investigated this [145]. We surmise that *in situ* gene editing of these innate immune cells can prevent this robust response or the effect can be mitigated through the use of immunomodulatory biomaterials carrying the edited cells. Moreover, advanced strategies such as whole genome sequencing (WGS) or exome sequencing would allow researchers to match donor cell lines to the recipient with the greatest compatibility as well as guide decisions on which HLA edits would further increase compatibility [145]. Finally, in an effort to reduce off-target edits with multiplex gene editing, one study has already begun to use base editors, which are characterized by their precision, on *B2M* to reduce the immunogenicity of chimeric antigen receptor (CAR)-T cells [165].

4.3.3. Organ xenotransplantation: Most severe cases may require the transplantation of an organ, as opposed to differentiated cells. The full potential of organ xenotransplantation is only realized when CRISPR/Cas9 editing technologies are used. Given that pig organ transplantation could initiate an immune rejection within minutes, CRISPR/Cas9 paves the way for knocking out genes known to cause an immune reaction or integrating genes that regulate immune and coagulation functions [166,167]. The field has found some success in knocking out α-1,3-galactose and porcine endogenous retroviruses, however, there is still abundant room for research before organ xenotransplantation can make its way to patients [168].

4.4. Gene editing for controlling protein dosages

Previous sections have explored the use of CRISPRa/CRISPRi to guide differentiation by promoting or repressing gene expression. While this technology proved to be effective in some applications in repairing tissue, in other applications, greater control and regulation are needed, which can also be achieved through other CRISPR technologies. For instance, certain applications, such as inflammation or dose-related diseases, require precise protein dosage control to sufficiently repair tissue rather than merely guiding differentiation down a particular cell lineage. In this section, we will review technologies to control protein dosages and their application to repairing damaged tissue.

4.4.1. Inflammation induced tissue degradation: Inflammation, though advantageous in its preliminary protection against infection and injury, is capable of producing environments that are particularly susceptible to tissue degradation and lack total repair. For instance, the result of inflammation caused by muscle or deep skin injury is fibrotic scarring, partially driven by inflammatory cytokines, which hinders complete tissue regeneration and heals by promoting fibrous scar tissue formation [169–171]. In another example, niche cells in the liver environment such as hepatic stellate cells (HSCs) become activated in response to hepatocyte injury, convert to myofibroblasts, and further the fibrotic cascade [172]. Moreover, chronic diseases such as arthritis and autoimmune diseases are characterized by elevated levels of proinflammatory cytokines which lead to accelerated tissue catabolism and impairment of resident stem cell niches [173–175]. Thus, the challenge here lies in total tissue regeneration in response to injury-induced inflammation. To mitigate these inflammatory consequences, many researchers have studied the use of anti-inflammatory molecules such as antifibrotic agents for muscle [176] and hepatic fibrosis [177] or anti-cytokine therapies [178,179]. Overall, the administration of these molecules is successful at counteracting the negative consequences of the pro-inflammatory cytokines, improving tissue repair, and reducing scarring. However, given the pleiotropic roles of pro-inflammatory cytokines and specifically their role in tissue homeostasis, complete inhibition of cytokines through these therapies would be detrimental to tissue repair. For instance, normal physiological levels of TFG-β1 are essential in tissue regeneration and remodeling and for reepithelization following injury [180,181] and IL-1α has been shown to mediate the stem cell niche by activating and proliferating cells [171,182,183]. The pleiotropic roles of these molecules suggest the benefit of an environmentally sensing and temporal anti-inflammatory biologic delivery system, which regulates the presence and persistence of these proteins. Thus, we can effectively regenerate tissue by either recruiting stem cells for proliferation and differentiation or by mitigating adverse inflammation.

Given the direct role of stem cells in regenerative medicine, the key to resolving tissue degradation from injury-induced inflammation resides in the activation of stem cells in the injured microenvironment. These cells hold to capacity to differentiate into a variety of tissue-specific cell types. Despite the success of stem cells in regenerative medicine, transplantation of stem cells in an inflamed resident environment has limited success. For instance, in a muscle injury environment that has elevated levels of TFG-β, injected muscle-derived stem cells (MDSCs) differentiate into fibroblasts, which limits the regenerative

capacity of the stem cells [184,185]. While these effects could be mitigated by the timing of the injection of the stem cells as there are time-varying degrees of inflammation post-injury [185], CRISPR/Cas9 knock-in offers even greater control to advance the regenerative potential of stem cells in these environments.

To enhance the regenerative potential of resident cells and prevent their differentiation into myofibroblasts, which furthers the fibrotic cascade, researchers have used CRISPRa/CRISPRi technologies to activate/repress genes to guide the desired differentiation. In one example, researchers encapsulated exosomes with CRISPRa technology and were delivered to HSCs to promote differentiation to hepatocytes [186]. Though this strategy significantly attenuated liver fibrosis it fails to target one of the root causes of fibrosis: inflammatory cytokines.

Through HDR-mediated knock-in, researchers have edited MDSCs that respond to TGF-β1-mediated inflammation [187] and iPSCs that respond to TNF-α and IL-1-mediated inflammation [188] by dynamically and dose-dependently translating biological antagonists to these cytokines. These stem cells behave as a closed-loop system, only delivering the antagonist when stimulated by an inflamed environment. In both studies, the target promoter for sgRNA was chosen for being stimulated and differentially expressed by the inflammatory cytokine. The studies were performed *in vitro* where the Cas9 plasmid, sgRNA, and cytokine antagonist transgene were transfected using lipofectamine transfection reagents. While this method of CRISPR/Cas9 delivery served its purpose for the *in vitro* studies, lipofectamine reagents are toxic and lower cell viability, making them an unfavorable reagent for translational studies [189]. Nonetheless, in this study, gene-edited MDCSs transplanted in a simplified muscle injury microenvironment with elevated levels of pro-inflammatory TGF-β1 had reduced differentiation down a fibrotic lineage, effectively reducing the post-injury fibrotic cascade [187]. Though the cells were not measured for their ability to differentiate into functional muscle cells as well as being tested in a complex muscle injury environment that has time-dependent inflammatory responses and many fibrotic stimuli, this study still shows promising progress toward tissue regeneration in an injury-induced inflamed environment [187]. In a similar study targeting TNF-α and IL-1-mediated inflammation, engineered iPSCs differentiated into chondrocytes and effectively reduced the inflammatory response, had decreased expression of degradative enzymes, and specifically the TNF-α-antagonist edited cells were resilient to cytokine-induced articular cartilage degradation [188]. Edited iPSCs with IL-1 antagonist expression were not sufficient to protect the articular cartilage in inflamed environments, as this engineered tissue suffered from suppression of ECM constituents in environments with elevated IL-1 [188]. This suggests the varying roles of many cytokines in an inflamed environment and the amount of the antagonist needed to see functional regeneration. Moreover, another important factor in these studies is properly determining the gene locus that the cytokine stimulates. NGS can be used to validate the simulation of a cytokine at a certain gene locus. To make use of CRISPR/Cas9's multiplexing potential, we can integrate antagonists for multiple cytokines or at multiple differentially expressed gene loci through multiplex HDR [190]. Using CRISPR/Cas9 in these studies, as opposed to traditional lentiviral gene delivery, allowed dosage precision and the creation of a regulated closed feedback loop.

4.4.2. Other applications in dosage-related diseases: In another attempt to regulate the expression of proteins, researchers integrated small molecule-assisted shut-off (SMASh) before an endogenous gene involved with differentiation using CRISPR knock-in [191]. The SMASh is dose-dependently regulated by NS3 protease inhibitors, indicating that the concentration of the protein of interest is regulated by NS3 protease inhibitor concentration [191]. This allows us to understand the dose-dependent role of endogenous proteins on cellular differentiation, which is critical for dosage-related diseases, such as FOXG1 syndrome which affects neuronal development, and for regulated differentiation to create tissue constructs. SMASh-mediated protein concentration can also be used in disease modeling as done in this study [191], and for therapeutic purposes to precisely deplete proteins that contribute to tissue degeneration, such as pleiotropic inflammatory molecules. However, this approach would be less advantageous than the HDR-mediated knock-in of cytokine antagonists since it lacks closed-loop delivery by relying on an external molecule—the protease inhibitor.

5. Gene editing as a research tool in regenerative medicine

In addition to the therapeutic applications of gene editing for regenerative medicine, many studies have used CRISPR technologies as a research tool to screen for genes and model diseases, which is a prerequisite for therapeutic applications. The precision of CRISPR technologies permits researchers to gain a fundamental understanding of target genes, cells, and the disease to guide translational research.

5.1. Genetic screening

CRISPR technologies have been used to screen genes, particularly genes involved in differentiation, for regenerative medicine applications. Researchers have used CRISPR knock-in and knockout for gain and loss of function studies, respectively. Moreover, researchers have knocked in fluorescent markers or epitope tags. These applications uncover the biological role of transcription factors and other proteins in driving directed cell differentiation, which can then be applied as a therapeutic target gene. These studies have elucidated many genes for their role in regenerative pathways, disease progression, and other various biological processes.

5.1.1. Loss and gain of function studies: As discussed in Section 4.2, many studies rely on CRISPRa/CRISPRi to induce the differentiation of stem cells down a certain cell lineage. The success of this application is contingent on proper knowledge of which transcription factors to activate and repress. In this section, we will review applications of CRISPR technologies to better understand the function of genes during cell differentiation. Specifically, we will focus on using CRISPR for loss or gain of function screening studies. Previous methods have relied on systematic trial-and-error with lentiviral transcription factor delivery, which is technically challenging, or a comparison of the expression profile of stem cells and mature cells, which has a high failure rate and is difficult to interpret (Fig. 5(a)). CRISPR technologies facilitate a precise understanding of genes, as well as the relationship of multiple genes involved in the same signaling pathway, using CRISPR’s multiplexing capacity.

Many studies explore the differentiation of stem cells to mature cells as mediated by transcription factors. To maintain proper osteogenesis, as well as for other organs' development such as the teeth and neural crest, the level of Msh homeobox 1 (MSX1), a homeobox transcription factor, must be maintained [192]. Ubiquitin ligases add ubiquitin molecules to proteins, making them susceptible to degradation, while deubiquitinating enzymes (DUBs) remove ubiquitin molecules from proteins, indicating that proteins are regulated by ubiquitinating and deubiquitinating activities. Both MSX1 and the balance of ubiquitinating and deubiquitinating processes have major roles in stem cell differentiation and development yet are not fully understood at a molecular level. In an effort to understand the factors affecting MSX1 protein regulation during stem cell differentiation, researchers have directed their attention to loss-of-function DUB studies [193]. Through genome-wide DUB CRISPR knockout, researchers were able to understand how and which DUBs promote human mesenchymal stem cell (hMSC) osteogenic differentiation by regulating *MSX1* expression [193]. Thus, such studies serve as valuable screens for understanding which DUBs, and the corresponding transcription factors, have promising regulatory roles that can be later used for engineering tissue constructs through CRISPRa and CRISPRi. In a similar study involving a genome-wide CRISPR screening, researchers used CRISPRa to activate multiple endogenous targets simultaneously to create genetic interaction maps to ultimately delineate factors involved in neuronal reprogramming (Fig. 5(b)) [194]. Similarly, in another study, researchers used CRISPRi on differentiated iPSCs to elucidate genes essential for neuronal survival as well as implications of gene knockdown on neuronal morphology [136]. Many other loss-of-function studies have explored the critical function of genes on organ development, controlling morphogenesis, cell fate determination, cell–cell communication, and cell adhesion, which all have important implications for regenerative medicine [195]. Other studies have used CRISPR technologies to investigate the regulatory roles of genes important for biological processes, such as learning and other brain functions [196,197]. In all these applications, it is critical to highlight the superiority of CRISPR technologies compared to other methods, such as RNAi, for loss-of-function studies.

In addition to using CRISPR technologies to uncover the function of a particular gene, it can also be used to understand the effects of a poorly understood mutation. For instance, the $STAT3^{K392R}$ mutation has formally been thought to be correlated with the immune system. However, one study elucidated the role of this mutation in dysregulating pancreatic differentiation, leading to pancreatic hypoplasia [198]. Moreover, CRISPR allows the effect of a mutation to be uncovered while disregarding any genetic artifacts. For instance, most studies compare healthy groups to patient groups to uncover the effect of a mutation. However, to precisely understand a mutation-specific phenotype while disregarding genetic differences, it is critical to include a corrected patient group, which can effectively be done through CRISPR. Finally, given its multiplexing nature, CRISPR has paved the way for understanding polygenic neuronal disorders [196].

5.1.2. Fluorescent lineage tracing: Researchers have also used CRISPR knock-in to incorporate a fluorescent marker or epitope tag to further understand the function of a gene, specifically within the context of regenerative medicine (Fig. 5(b)). These reporters are valuable tools as they allow real-time observation of gene expression dynamics, cell lineage

tracing, and isolation of a specific cell population, as well as for useful identification when transplanted *in vivo*.

To better understand the mechanisms behind axonal injury and retinal ganglion cell (RGC) death resulting from optic nerve injury, researchers have created CRISPR-engineered fluorescent reporter stem cell lines [199]. These cells underwent an mCherry CRISPR knock-in in the endogenous *Brn3b* ORF and were differentiated into RGCs, followed by purification using cell sorting through fluorescence-activated signals [199]. This strategy is especially useful for cells that are difficult to differentiate. Similarly, to explore if *Wnt4* expression is sufficient to induce β-cell maturation in stem and progenitor cells, researchers used CRISPR knock-in to add the GFP sequence before the insulin promoter [200]. It is critical to consider the locus of the knock-in, as some sites are prone to transient protein expression, indicating loss of fluorescent protein expression during the timespan of differentiation [201]. AAVS1 is one of the few loci that allow for stable and long-term transgene expression [201].

The fluorescent marker is typically in the ORF of an important mature cell marker or transcription factor. The fluorescent marker may be fused to the target protein post-translation, which is useful for protein intracellular localization, protein kinetics and dynamics, and protein–protein interactions [202]. However, the fused fluorescent reporter may affect the function of the target protein. To mitigate this, a self-cleaving peptide may be added to prevent the fluorescent tag from fusing to the target protein as they are translated independently.

5.2. Creating disease models

Drug development from conception to market is fraught with complexities, not least of which are the challenges presented by the reliance on animal studies. The crucial issue is the physiological and genetic chasm that separates humans from the animals used in research. This disparity can lead to significant differences in how a drug behaves within the body, sometimes rendering findings from animal studies misleading when applied to humans [203]. Drugs that promise efficacy and safety in animals may falter when faced with the intricacies of human biology, as animal models don’t fully represent human diversity, including factors like gender, age, race, and disease conditions (Fig. 5(c)). Beyond the scientific limitations, the ethical landscape surrounding animal research is contentious. The welfare of animals and the moral implications of subjecting them to experimental treatments motivate a growing call for alternative research methodologies.

In pursuit of more ethical and effective drug development, the scientific community is turning to advanced alternatives such as organ-on-a-chip, organoids, computational modeling, and human cell-based assays [204]. These innovations offer insights into human physiology and disease more relevantly than animal models, enhancing the efficiency and accuracy of drug discovery processes. Gene editing enhances this shift, enabling the creation of tissues and organoids from iPSCs, including those derived from patients (Fig. 5(d)). This approach not only circumvents ethical issues associated with animal testing but also leads us toward a future where personalized and regenerative medicine is accessible and effective

for everyone, marking a significant advance in our ability to treat and understand human diseases.

Organ-on-a-chip models are *in vitro* microfluidic devices lined with cells perfused by relevant physiological flows, designed to replicate the complexity of organs and in some cases the interactions between organs [205]. These models are used to study diseases, develop drugs, and advance personalized medicine. Researchers have created models for the heart, liver, bone, brain, and the blood–brain barrier. As such, researchers successfully modeled Alzheimer's disease, hepatitis B virus, and the release of dissolved metals from arthroplasty implants [206–208]. Combining gene editing technologies with these models has allowed researchers to develop personalized models, necessary for understanding disease pathology and treatments for each patient. Table 2 [209–215] highlights successful organ-on-a-chip and organoid models and how they have used CRISPR/Cas9 to model diseases and assess treatments. These models are invaluable tools to accelerate the field of regenerative medicine.

5.2.1. Organoid models: The generation of iPSC-derived organoids involves reprogramming somatic cells into iPSCs, which are then cultured in a 3D environment and exposed to specific growth factors and signaling molecules. These orchestrate a tightly regulated differentiation process, ultimately leading to the formation of organoids that recapitulate the structural and functional characteristics of human organs. Organoid development can vary depending on the organ being modeled. While some like intestinal organoids can form within days, complex organs like the brain may take months to mature [216].

Significant hurdles remain before the full potential of organoid technology can be realized. One challenge lies in achieving precise spatiotemporal control during organoid development. Precise orchestration of growth factors and signaling molecules is crucial for guiding stem cell differentiation into the desired cell types and recapitulating the intricate architecture of real organs. Researchers are actively developing bioengineering strategies to achieve this spatiotemporal control [217]. For example, microfluidic devices allow for the controlled delivery of nutrients, growth factors, and signaling molecules to organoids, enabling precise manipulation of the organoid's microenvironment over time. This approach can create gradients of biochemicals that mimic the natural development cues of organs [218]. Another approach leverages 3D bioprinting technologies, which enable the deposition of cells and biomaterials in defined spatial arrangements, allowing for the construction of organoids with organized structures that closely mimic the architecture of native tissues [219–222]. Techniques like photopatterning and optogenetics offer dynamic control over cell behavior by using light to activate or inhibit specific cellular pathways. These methods can be used to spatially and temporally control cell differentiation and organization within organoids [223]. Another approach is magnetic manipulation. Applying magnetic fields to manipulate cells or biomaterials embedded with magnetic nanoparticles allows for the non-invasive assembly and patterning of organoids. This method can be used to spatially organize cells into complex structures without physical contact [224,225]. The ability to introduce specific mutations or disease-causing genes using gene editing is a significant strength of organoids. Moreover, gene editing facilitates the opposite scenario as well—existing

disease-causing mutations can be corrected to study the resulting organoid (Fig. 5(d)). This allows researchers to model human diseases with greater fidelity compared to traditional animal models. Patient-derived organoids (PDOs) further enhance this advantage, enabling the study of personalized disease mechanisms and individual responses to potential therapies (Fig. 5(d)). We will explore advancements in organoids for the retina, brain, lungs, and ovaries, and opportunities for gene editing to advance these models.

5.2.1.1. Retina. Organoids offer a promising avenue for elucidating the pathophysiology of ocular diseases, especially given the escalating prevalence of retinal disorders and the limitations of rodent models in recapitulating human ocular physiology. The retina, affected by degenerative conditions such as glaucoma, X-linked juvenile retinoschisis (XLRS), and retinitis pigmentosa, serves as an exemplary model for regenerative medicine due to its direct relevance to both ophthalmological and broader neurological research [62,226]. Retinal organoids, particularly those modeling diseases like retinitis pigmentosa—often caused by single-gene autosomal recessive mutations—provide a streamlined system for employing CRISPR/Cas9 gene editing techniques. This simplicity facilitates the exploration of CRISPR/Cas9's therapeutic potential and its delivery mechanisms within a controlled environment.

Advancements in retinal organoid research are illuminating new pathways for tissue engineering, especially in the realm of transplantation therapies. Investigations into the integration of hPSCs for reconstructing the retinal pigment epithelium (RPE) highlight potential treatments for conditions such as geographic atrophy, which precipitates photoreceptor cell death [227]. Nevertheless, challenges persist, including the organoids' lack of vasculature, the intricate architecture of the RPE cell layer, missing components of photoreceptors due to disk shedding, an absence of connectivity with the RPE, and insufficient cell turnover, which collectively impede the accurate modeling of retinal diseases. Additionally, the reduced mimicry of photoreceptor interactions and the dynamic processes inherent to retinal biology limit the fidelity of disease models and the development of potential therapeutic transplants [226].

Despite these hurdles, retinal organoids have successfully modeled key developmental phenomena, including cell organization and photoreceptor morphogenesis, while also uncovering potential therapeutic targets. For instance, research has elucidated the role of *RPGR* mutations in X-linked retinitis pigmentosa, revealing a pathogenic interaction between RPGR and Gelsolin that facilitates actin polymerization [228]—a process that can be inhibited by specific drugs, with organoids serving as a validation platform. However, the complexity of replicating *in vivo* cellular interactions *in vitro* remains a formidable challenge, particularly due to the critical interplay between retinal and brain functions. This complexity necessitates the development of co-culture systems that integrate retinal and brain organoids, though such systems present variability in organoid characteristics and demand extensive maintenance.

5.2.1.2. Brain. Human brain development is a complex orchestration of molecular and cellular processes that evolve through intricate processes, forming the brain's complex structures and functions. This development is guided by genetic blueprints and influenced by

environmental factors across various spatial and temporal scales, including cell cycles, cell interactions, and morphogen gradients. Understanding the architecture and mechanisms of both healthy and disease-associated brain development is paramount, yet challenges such as species-specific differences in animal models and the inaccessibility of developing human brain tissues limit our understanding. Brain organoids have emerged over the last decade, offering models that mimic the cellular composition and architecture of the developing brain [229–231].

Brain organoid development uses either unguided or guided methodologies. Both begin with the formation of embryoid bodies (EBs) from iPSCs. Unguided approaches rely on self-organization under specific culture conditions to produce organoids with various brain region identities, while guided methods apply patterning factors to direct the development of region-specific organoids, such as those of the forebrain or cerebellum. These organoids approximate the developing human brain in cellular diversity, architecture, and developmental trajectory, with transcriptome analyses revealing similarities to fetal brains and even postnatal development stages.

Brain organoids have been used to model a wide range of neurological disorders, including neurodegenerative diseases, neurodevelopmental disorders like autism spectrum disorder (ASD) and lissencephaly [232–234], Rett and Miller-Dieker syndromes [235,236], schizophrenia [237], viral infections [238–240], and environmental exposures [241]. One study found that CRISPR/Cas9 could correct cerebral organoids derived from iPSCs with the *HEXB* mutation for Sandhoff disease, a neurodegenerative disorder [211,242]. However, a major challenge in deciphering disease mechanisms using these models is filtering out interindividual genetic variation, often referred to as “background noise.” Traditionally, reducing this noise relies on increasing sample size, which significantly increases cost and workload. Using gene-edited hPSCs for organoid generation is one feasible solution for this concern.

Another solution emerges from the development of human brain chimeroid technology [243]. This technique bypasses generating individual diseased organoids from each patient’s iPSCs. Instead, it co-develops mature organoids by reaggregating neural stem cells or committed progenitor cells from multiple single-donor organoids. These chimeroids effectively capture donor-specific responses to environmental toxins, offering a scalable platform for high-throughput assessment of genetic or neurotoxic effects across diverse individuals.

5.2.1.3. Lungs. Because of the nature of respiratory diseases, it is important to observe host–pathogen interactions and the progression of infection in relevant models. Studies have examined pulmonary fibrosis, pulmonary tuberculosis, and influenza viruses using alveolar organoids [244]. Lung organoids can replicate the branching structures and capillary network missing from two dimensional (2D) cultures. Furthermore, these disease models allow researchers to screen antiviral drugs, measuring the rate of infection and the drug’s antiviral capacity. Lung organoids can also portray the immune response from cells, such as macrophages [245]. Like the retinal and brain organoid models, these models lack the inclusion of sufficient vasculature. Upon further advancement, these organoids will be

invaluable for drug screening and toxicity studies, and eventually, they will provide an avenue for precision medicine with PDOs [244].

5.2.1.4. Ovaries. Hormonal changes, including pregnancy, menopause, and the menstrual cycle, affect ovarian function and all have a temporal element. Thus, ovarian organoids would be beneficial for these applications. Current research supports that these models can identify targets for disease treatment, including infertility, endometriosis, and ovarian cancer [246]. The next advancement will be integrating hormonal secretion within ovarian organoids to create a model that changes over time. Ovarian organoids also lack the multiorgan environment central to the reproductive system. The most successful and reliable use of ovarian organoids has been as tumor models. PDOs have been used to screen chemotherapeutics for the most effective ovarian cancer treatments. However, PDOs pose the challenge of inter-individual variation, indicating results cannot be broadly applied to all patients [247]. However, this concern can be mitigated through the use of organoids derived from gene-edited stem cells to control genetic differences between patients. In addition to chemotherapeutic screening, organoids can solidify relationships between genotype and phenotype by controlling gene expression with CRISPR technologies. Completing whole-genome screening on edited cells will generate a large collection of data establishing connections between genes and diseases [242].

The application of gene editing to retinal, brain, lung, and ovarian organoids creates relevant disease models for gaining deeper insights into molecular mechanisms, and for screening both small molecular drugs and biologics. Gene editing can also help these models overcome inter-individual variation. These models are invaluable in the field of regenerative medicine, offering frameworks to regenerate damaged and diseased tissues. However, further refinement in replicating the intricate cellular and physiological context within organoids is essential for realizing their full potential in drug discovery and regenerative medicine.

5.2.2. Animal models: Gene editing tools can be applied to animal models to replicate disease and investigate therapeutics. Previous animal models have mimicked diabetes, cardiovascular diseases, and immunodeficiencies, among others [248]. Choosing the optimal species is a challenging aspect of this method and requires a balance of anatomical and physiological characteristics that are similar to humans and realistic for the research needs. It is important to consider which organ systems are of focus, the lifespan, and the feasibility of gene editing in that species. Rodents, such as mice, are used in the majority of animal models because they are easier to genetically engineer, have a short lifespan, and are relatively simple to maintain. However, there are no species that are entirely translatable to humans. Rodents cannot consistently represent human systems and pathways, leading to a higher clinical failure rate from drug testing. The sheep model has been applied to a wide range of disease physiologies, including the nervous system, cardiovascular diseases, and musculoskeletal disorders. Moreover, sheep have a longer gestation period of 150 days, providing an opportunity to study fetal regeneration. Although horse models are more costly, they are beneficial for survival studies observing musculoskeletal diseases and injuries [249]. Yet, larger animals raise ethical issues deterring the broad acceptance of their use, and they can be more difficult to genetically manipulate and maintain. While it is unlikely

that scientific studies will ever wholly eliminate animal use due to their unique benefits, developing non-animal disease models will reduce unnecessary animal use. Eventually, organoid and organ-on-a-chip models could replace animal models in cost, versatility, control, and reliability.

6. Future work

After years of development since its discovery in 2012, CRISPR technologies have gone through many advancements, from simple DNA cleaving now to base conversions, transcriptional regulation, RNA editing, and advanced knock-in. In Sections 4 and 5, we have discussed the application of these advancements to regenerative medicine, in the context of therapeutic applications and as a versatile research tool. Nonetheless, there still lies ample work to fully realize the use of CRISPR technologies in regenerative medicine. Fig. 6 summarizes some strategies currently being used to resolve the challenges.

Regarding the ethics and legal implications of gene editing, the critical factors include the type of cells being edited, the long-term consequences, and the purpose of the editing. For this review, none of the studies mentioned rely on germline editing for regenerative medicine applications. This aligns with the recently signed global moratorium and the stance of groups like the Alliance for Regenerative Medicine, which disapproves of current clinical uses of germline editing [250]. This precaution safeguards against unsafe and ineffective germline mutations using today's technologies, which still require further improvements. With somatic gene editing for therapeutic purposes, patients can rest assured that the intervention will require informed consent, will not result in any heritable DNA modifications, and ultimately seeks to treat the disease. While germline gene editing may prove advantageous by preventing children from being born with severe genetic diseases, its efficiency, and safety are critical considerations, alongside preexisting moral and psychological concerns. That being noted, germline editing remains a prospective avenue within the gene editing field, including its potential applications in regenerative medicine.

6.1. Enhancing CRISPR delivery with temporal and spatial control

One of the main limitations of gene editing is its delivery, primarily in *vivo* circumstances. Excluding *ex vivo*-specific methods such as electroporation, viral vectors, and non-viral methods pose their own limitations which emphasize the need for CRISPR delivery that has both temporal and spatial control. Often, the benefits of one delivery system are typically the limitations of another, and vice versa, limiting the clinical opportunities of *in vivo* gene editing.

Regarding spatial control and a reduction of *in vivo* off-target editing, more research should be done enhancing both polymeric and lipid-based delivery systems. For polymeric or metallic nanoparticles encapsulating gene editing components, researchers can conjugate tissue-specific peptides or CPP to enhance delivery [251]. Moreover, the intersection between biomaterials and gene editing can be leveraged to take advantage of materials that respond to certain stimuli or to recreate the microenvironment of the target cell type. For instance, researchers have developed laser-controlled delivery of CRISPR/Cas9 using

gold nanoparticles [252] or near-infrared responsive nanovectors to release CRISPR/Cas9 [253]. In another study, researchers used scaffolds mimicking the BM microenvironment loaded with a chemoattractant and RNPs to attract and edit leukemia stem cells [254]. These applications showcase the temporal and spatial control researchers have achieved using novel biomaterials. Nonetheless, the adoption of biomaterials into clinical trials remains very slow in comparison to CRISPR delivery using viral vectors due to their poor editing efficiency, though some have achieved higher editing efficiency than the commercial standard, Lipofectamine CRISPRMAX [255,256]. For lipid nanoparticle-mediated delivery, one study reported that by simply adding an additional lipid to the four preexisting components, the internal charge adjustment modified the tissue-specific delivery to reach epithelial cells, B cells, T cells, lung basal cells, and hepatocytes [257,258]. However, with the abundance of opportunities for design and parameters to fine-tune, such as ligand conjugation, physical properties, chemical properties, lipid molar ratios, and environmentally responsive properties, there also is a need for efficient, high throughput testing of large variations in formulations. By enhancing the spatial and temporal control of CRISPR technologies using non-viral biomaterials and nanoparticles, its benefits may outweigh its poor editing efficiencies, proving these carriers as the stronger vehicle compared to their viral counterpart.

6.2. Optimizing CRISPR knock-in

Many studies we presented rely on CRISPR knock-in specifically through HDR to insert an exogenous sequence. However, HDR poses limitations regarding its timing, size, donor template design, and ultimately efficiency. For instance, in Section 4.1.1, we discussed that the knock-in of the *CFTR* gene in CF patients is limited by the repair pathway's poor efficiency. Previously, methods have relied on synchronizing the cell cycle through chemical inhibitors that capture the cells at the S and G2 phases and subsequently deliver CRISPR technologies [259]. However, these methods are technically challenging and lack translatability. Many researchers have studied modifications to the donor template, such as the length of the homology arms, the distance between the cutting site and the modification, single vs double-stranded donor DNA, and chemical modifications [260,261]. Moreover, blocking mutations have been incorporated into the donor template to prevent Cas9 recutting, as well as delivery of RNPs as opposed to pDNA, all to improve HDR efficiency [262]. Other work has explored using transient inhibitors to prevent the NHEJ and microhomology-mediated end joining (MMEJ) repair pathways to better facilitate the HDR pathway [263,264]. Moreover, the field has explored the synergistic efficacy of using small-molecule drugs in combination with CRISPR technologies to enhance knock-in efficiency [265]. All these parameters and adjustments create a host of design specifications that must be considered to maximize HDR efficiency. Future efforts should be directed towards creating a library screening these parameters and ultimately consolidating these design restrictions to create tools that can be used for a broad range of applications. Moreover, the advent of prime editing could serve as a replacement for HDR-mediated editing. Prime editing offers substantially higher editing purity than HDR, allows for a broad range of edits to be performed with high precision, and lacks a restrictive PAM sequence requirement near the target site [266]. While the editing efficiency of the original prime editing systems is not consistently high, advances to these systems such as through engineered pegRNAs have

led to breakthroughs in resolving many of these concerns [267]. The improvements in HDR efficiency as well as the advancement of prime editing facilitate gene knock-in, which is the required edit in many tissue engineering and regenerative medicine applications.

6.3. Reducing off-target editing

Off-target edits are a real drawback of the CRISPR/Cas9 technology and pose serious safety concerns. Many computational methods including in-silico tools and deep learning models have been designed to identify sites of off-target binding, such as DISCOVER-Seq^{+}, as well as to predict sgRNA efficiency, such as CRISPRon [35,268–270]. The use of such deep learning models in the field is rapidly evolving and still suffers from requiring large amounts of data to be reliable [271]. To mitigate this concern, some researchers have employed transfer learning to first train a model on a larger dataset, which is then adapted to a smaller dataset [272].

Moreover, the availability of the target gene based on its chromatin context is an important factor in editing efficiency. If the target gene is inaccessible, due to falling within a nucleosome dyad for instance, the endonuclease cannot locate the target gene [273]. Some researchers have employed features engineering into their machine learning models to account for accessibility factors and improve the reliability and performance of their model [274]. These features include epigenetic status and chromatin accessibility, microhomology properties, and DNA shape, and the inclusion of the latter feature was found to significantly improve predictive performance [275,276]. Overall, the inclusion of features that represent the biological context of the cleavage site into machine learning models has led to success in the predictive capabilities of these models and should be included to reduce off-target effects.

While many of the discussed solutions to off-target effects entail computational screening methods, there are other biological methods to reduce off-target effects. This can be done by either modifying the sgRNA and/or the endonuclease or by switching to RNP delivery rather than pDNA which reduces cell exposure to nucleases. The GC content, length, truncation, and chemical modifications of sgRNA can increase on-target activity and reduce off-target effects [33]. Recently, studies found that introducing short nucleotide extensions to the 5’ end of the gRNA spacer, effectively creating a hairpin structure, decreases off-target effects due to insufficient R-loop formation leading to the inactivation of Cas9 [277]. When combined with a screening method to identify top-performing extension sequences, researchers resolved concerns over inhibition of on-target activity and increasing off-target effects using their technology [278]. Moreover, novel Cas9 variants have found higher on-target specificity by targeting alternate, broader PAMs [279]. A combined approach that leverages both expansive computational methods to validate sgRNA targeting with *in vitro* screening to confirm model results will be required to create an all-in-one editing system with limited off-target effects.

6.4. Other avenues of future work

The mentioned directions for future work are not comprehensive, as much effort is required to enhance gene editing—specifically gene editing for regenerative medicine. One study

mentioned injecting gene-edited stem cells before inducing injury in the animal model, which we believe to be a highly effective application of gene editing for regenerative medicine [142]. This type of application, loosely classified as preventative medicine, can strengthen an individual's resident cells and tissues, thus mitigating the risk of future disease. This has promising, prophylactic outlooks in many monogenic or polygenic diseases, can be used to strengthen an immune-compromised immune system, or can strengthen an individual's stem cells and their differentiation potential before injury.

7. Conclusions

CRISPR technologies present a versatile and specific tool for gene editing that has been applied to the field of regenerative medicine. Through CRISPR, we have been able to repair diseased tissues as well as tissues susceptible to degradation. Moreover, we can screen the functions of genes and create relevant disease models. The myriad of CRISPR technologies that have been fine-tuned throughout the years have increased the technology's specificity and efficacy. In this review, we present these technologies and their limitations, which highlight the future work needed to realize the full translational potential of developing specific, safe, reliable, and effective gene editing therapies for tissue regeneration.

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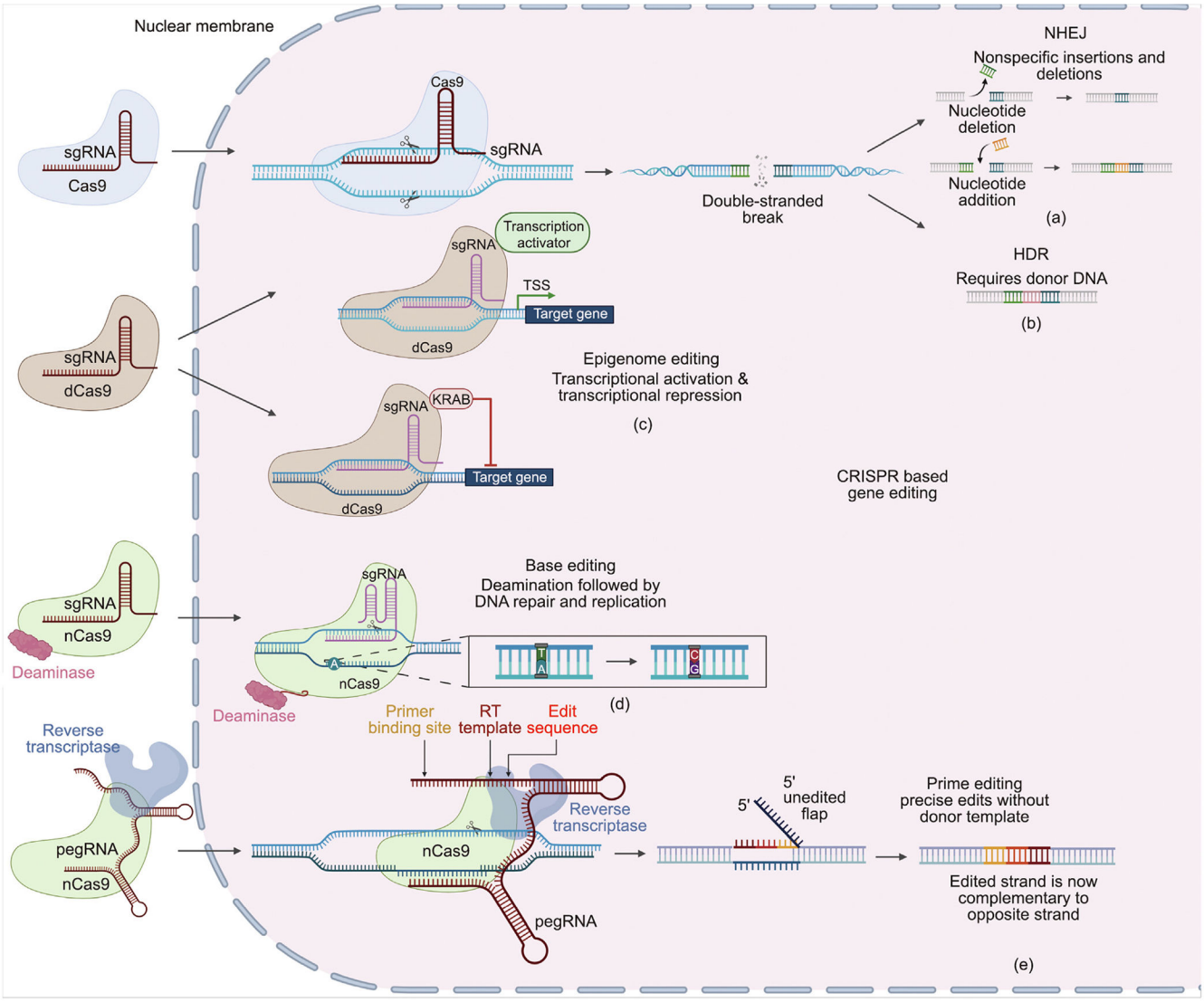

Fig. 1.

Different types of CRISPR systems and repair pathways within the cell nucleus. (a) NHEJ-mediated editing relies on nonspecific insertions and deletions at the DSB site using a Cas9 enzyme guided by single guide RNA (sgRNA). (b) HDR-mediated editing requires the delivery of a donor template with sequence-specific homology to insert at the DSB site using a Cas9 enzyme guided by sgRNA. (c) Epigenome activation and repression using a deactivated Cas9 enzyme fused with transcriptional activators or repressors. (d) Base editing using a Cas9 nickase fused with an adenosine or cytidine deaminase and uracil glycosylase inhibitor (UGI) for deamination of cytosine. (e) Prime editing using a Cas9 nickase fused with a reverse transcriptase, guided by pegRNA. RT: reverse transcriptase; nCas9: nicked Cas9.

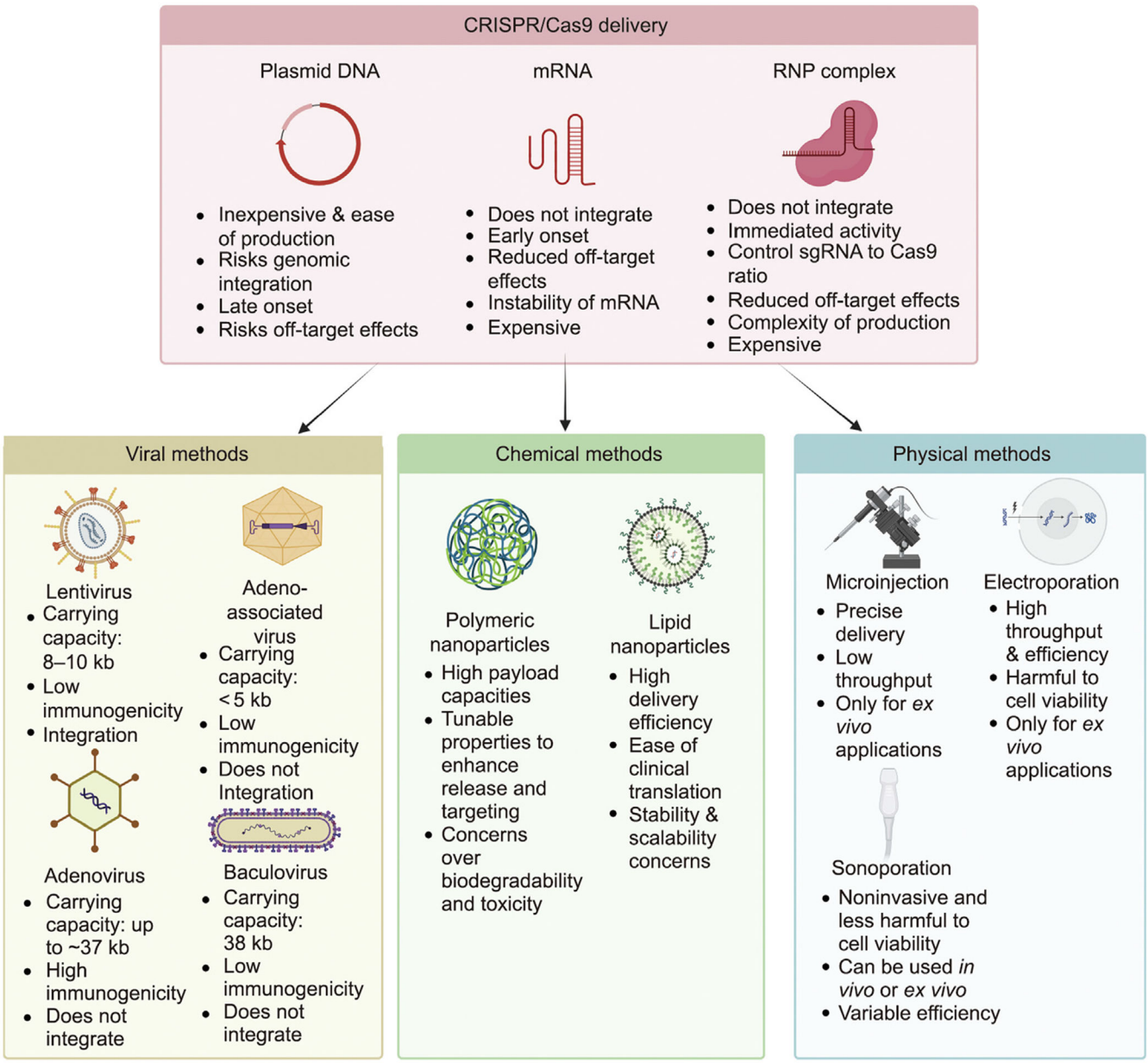

Fig. 2.

Summary of *in vitro*, *in vivo*, and *ex vivo* CRISPR/Cas9 delivery methods. Viral, chemical, and physical methods are highlighted with their respective advantages and limitations. RNP: ribonucleotide; mRNA: messenger RNA.

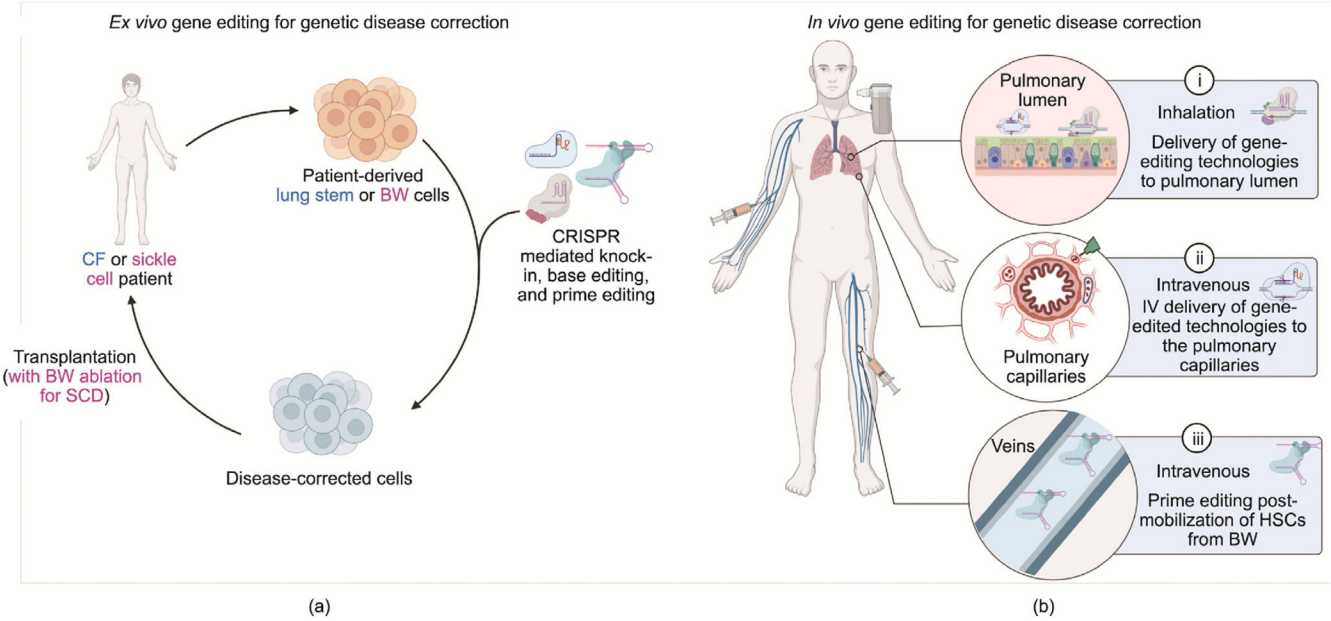

Fig. 3.

Summary of *ex vivo* and *in vivo* gene editing for CF and SCD. (a) Stem cells are harvested from the lung and bone marrow (BM) of diseased patients. CRISPR technologies are delivered to cultured cells by physical, chemical, or viral methods. Cells are transplanted back to the patient, with or without cell ablation to remove diseased cells. (b) For (i and ii) CF, inhaled or systemically delivered technologies edit the airway epithelial tissue. For (iii) SCD, hematopoietic stem cells (HSCs) are mobilized from the BM to the peripheral bloodstream for systemic administration of CRISPR technologies. IV: intravenous.

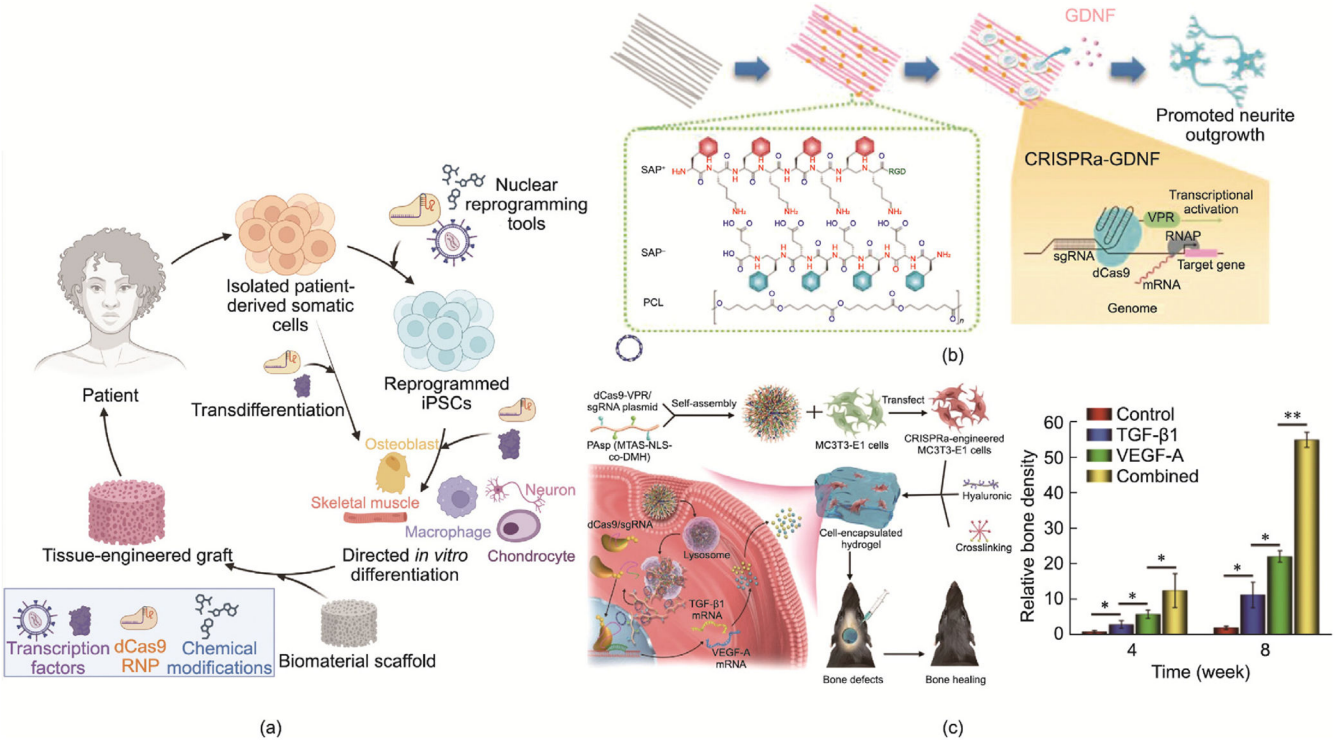

Fig. 4.

Gene editing to augment tissue repair. (a) The cycle of stem cell editing and transplantation. Somatic cells are harvested from a patient and transfected with nuclear reprogramming technologies. Technologies include chemicals, viral transcription factor delivery, or CRISPR RNP (or nucleic acid) delivery. Subsequent iPSCs may be differentiated *in vitro* into specified cell types using CRISPR-mediated epigenome editing or viral transcription factor delivery. Cells may be transplanted using biomaterial-based scaffolds in patients. CRISPR technologies have also been used for direct transdifferentiation between two mature cell types. (b) Novel biomaterials can carry and non-virally deliver CRISPR components. Self-assembled peptide (SAP) coating on polycaprolactone (PCL) nanofibers forming SAP-coated scaffolds to load and release CRISPRa components for neuronal differentiation. Reproduced from Ref. [119] with permission. (c) Cell-encapsulated hydrogels are used to implant CRISPRa-engineered cells into regions with bone defects (left). A combination of CRISPRa targeting *TGF-β1* with *VEGF-A* yielded the most improvements in bone density (right) and ultimately better bone healing. Reproduced from Ref. [132] with permission. *$p < 0.05$, **$p < 0.01$. GDNF: glial cell-derived neurotrophic factor; VPR: potent tripartite activator, VP64, p65AD, and Rta; TGF-β: transforming growth factor beta; VEGF-A: vascular endothelial growth factor A.

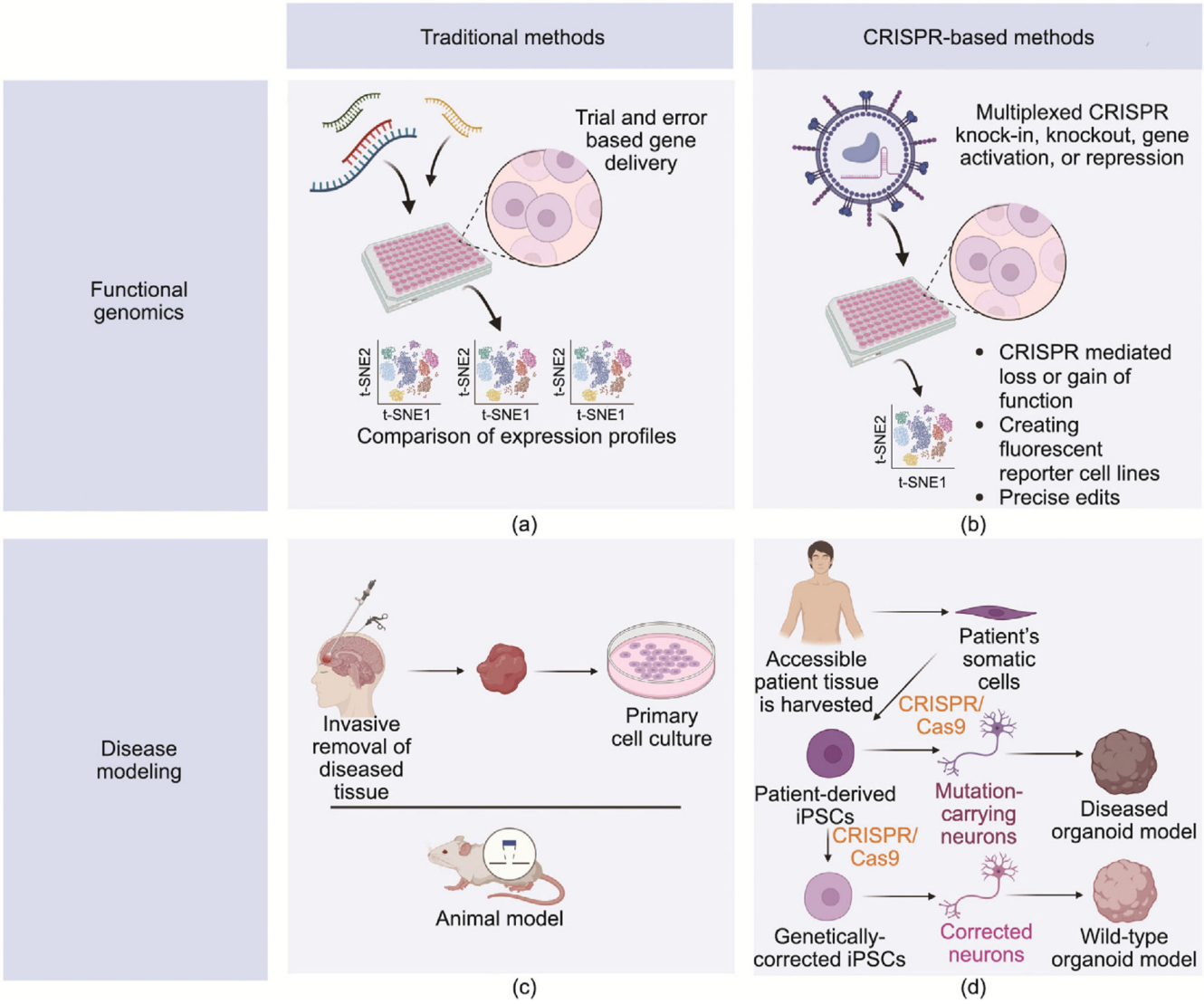

Fig. 5.

Comparison of traditional methods vs CRISPR methods for genetic screening and disease modeling. (a) Traditional methods for studying the function of genes, specifically for their role in differentiation, rely on randomized trial-and-error delivery of transcription factors to stem cells and a comparison of expression profiles in mature, stem, and diseased cells. (b) CRISPR methods study the function of genes, specifically involved with differentiation, by knocking in and knocking out a particular gene as well as inserting fluorescent markers before a gene of interest. (c) Traditional methods for disease modeling rely on the invasive removal of the diseased tissue, which typically requires surgical intervention, followed by a primary cell culture. (d) Somatic tissues are harvested from accessible, noninvasive sites in an individual, creating a culture of primary somatic cells. CRISPR is used to reprogram and correct these cells to create highly specified organoid models.

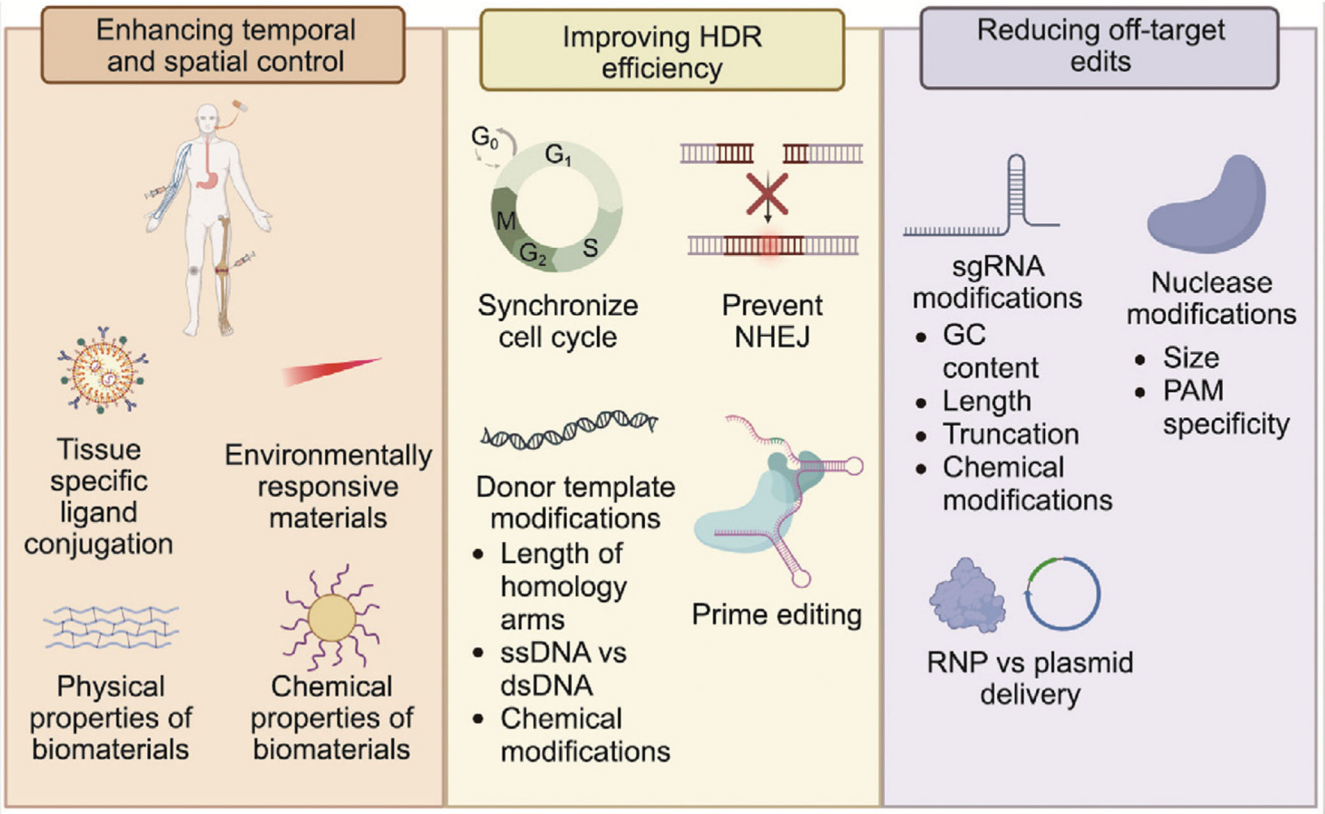

Fig. 6.

Future avenues of work for enhancing gene editing for regenerative medicine applications. Discussed avenues include enhancing temporal and spatial control for the delivery of CRISPR components, reducing CRISPR off-target edits, and improving HDR efficiency.

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Table 1

Summary of notable gene-editing approaches to prevent post-transplantation immune response.

Target gene	Gene edit and/or transfection	Cell type	Purpose	Impact on immune response	Limitations	Refs.
HLA-B	CRISPR knockout	iPSCs	Create immunocompatible HLA-homozygous iPSC cell line banks for ease in stem cell allotransplantation	Prevented HLA-induced graft rejection while maintaining stemness and differentiation capacity	Altering the HLA profile affects NK cell response Poor efficiency with many variations of indels and clones	[153]
HLA-A, HLA-B, and HLA-II (*CIITA*)	CRISPR knockout	iPSCs	Increase iPSC donor compatibility through HLA-C retention and decrease the number of iPSC lines required to be immunologically compatible with most of the world's HLA haplotypes	Suppressed HLA-induced and NK-mediated graft rejection while retaining some HLA expression for antigen presentation	Additional HLA retention may be required to suppress NK cell activity in some individuals with heterogeneous HLA-C1/HLA-C2 haplotypes Off-target mutagenesis and risk of tumorigenicity must be assessed	[156,157]
HLA-I(*B2M*), *HLA-II* (*CIITA*), *PVR*, and *HLA-E*	CRISPR knockout and lentiviral gene transduction (*HLA-E*)	iPSCs	Create expansive allogenic cell sources that escape recognition by alloreactive host CD4 and CD8 T cells and host $CD94^+$ $NKG2A^+$ and $DNAM\text{-}1^+$ NK cells	Cells exhibited longer survival both *in vitro* and *in vivo*	Multiple gene edits increase the likelihood of sources of error. The study would benefit from multiplexed editing	[158]
MHC-I (*B2M*), *MHC-II* (*CIITA*), and *CD47*	CRISPR knockout and lentiviral gene transduction (*CD47*)	iPSCs	Generate fully functional, hypoimmunogenic stem cells and their differentiated derivatives *CD47* will prevent IFN-γ release from NK cells	Stem cells differentiated into ECs, smooth muscle cells, and cardiomyocytes all evaded immune rejection in fully MHC-mismatched recipients and had long-term survival	Hypoimmunogenic pluripotent stem cells may present long-term concerns over malignant transformation or diminished virus clearance	[152]
HLA-A, *HLA-B*, and *HLA-II* (*CIITA*)	CRISPR knockout	iPSCs	Simultaneous knockout using HLA-homozygous iPSCs to minimize immune rejection caused by HLA mismatches, while retaining HLA-C	Manufactured hypoimmunogenic iPSCs	Off-target/undesired edits between *HLA-A* and *HLA-B* regions lose iPSCs' ability to differentiate into cardiomyocytes Required to match HLA-C haplotype with donors HLA editing resulted in large deletions, chromosomal translocations, and complex genomic rearrangements The low success rate of clones with desired edits	[151]
HLA-I (*B2M*), *HLA-G1*, and *HLA-G5*	CRISPR knockout, knock-in, and lentiviral transduction, respectively	hPSCs	Block HLA-I to prevent CD8 T cell-mediated killing and utilize HLA-G given its broadest lymphocyte-targeting spectrum to avoid rejection from other immune cells	Inhibited T cell-, NK cell-, and APC- related immune rejection	The tumorigenic potential of the hypoimmunogenic cells needs to be assessed	[161]
HLA-I (*B2M*) and *HLA-II* (*CIITA*)	CRISPR knockout	Human endothelial colony-forming cells	Use non-immunogenic, allogeneic ECs to construct vascularized and perfusable grafts	Graft did not elicit immunogenic NK cell, CD4, or CD8 T cell response Committed progenitors are less likely to give rise to teratomas ECs retained vasculogenic potential	Committed progenitors lost their ability to differentiate into many cell types	[162]
MHC-I (*B2M*), *MHC-II* (*CIITA*), and *CD47*	CRISPR knockout and lentiviral gene transduction (*CD47*)	iPSCs	Creating non-immunogenic iPSC-derived ECs for the treatment of cardiovascular and pulmonary diseases	Grafts survived and treated diseases in immunocompetent and fully allogeneic recipients	Concern over large-scale manufacturing	[147]

Table 2

Organoid and organ-on-a-chip models for elucidating disease pathology, advancing drug discovery, and enhancing regenerative medicine research.

Model type	Organ	Cell source	CRISPR delivery	Function of CRISPR/Cas9	Model fabrication	Functional results	Ref.
Organoid	Retina	Patient-derived iPSCs	RNP complex and ssODNs delivered via electroporation for HDR-mediated editing	Correction of *LCA5*-associated retinal disease	Differentiated corrected, patient-derived cells into 3D retina-like cells	Confirmed the rescue of lebercilin expression and localization	[209]
		Human embryonic stem cells (hESCs)	Separate lentiviral transduction of Cas9 and guide RNA	RPE-specific *Vegfa* knockout to regress choroidal neovascularization	After 130–160 days of differentiation, organoids were selected for lentiviral transduction	Successfully achieved RPE-specific editing system, useful for creating targeted cell therapies	[210]
	Brain	Infantile patient-derived iPSCs	A plasmid containing gRNA and Cas9 with separately delivered ssODN was electroporated into cells	Correction of *HEXB* mutation for Sandhoff disease	Organoids are generated using corrected, patient-derived cells	Decreased levels of GM2 ganglioside and cell proliferation compared to diseased organoids	[211]
	Lung	Bona fide hESCs	Cells were treated with plasmid Cas9 and guide RNA using electroporation	Model fibrotic lung disease by introducing several mutations in *HPS* genes and IL-11	Mutated cells were ultimately differentiated into the vAFE under 3D suspension culture conditions	*HSP1*, *HSP2*, and *HSP4* mutations recapitulated diseased phenotype Identified IL-11 overexpression as a fibrotic marker	[212]
	Pancreas	Patient-derived pancreatic ductal adenocarcinoma tissue	Lentiviral transduction of CRISPR/Cas9	Introduce additional types of mutations to diseased organoid models to understand sensitivities and correlations	Diseased organoids were resuspended as single cells, transduced, and selected to obtain clonal organoid lines	Understand drug–gene interactions to screen drugs comprehensively	[213]
Organ-on-a-chip	Heart	Patient-derived iPSCs	Transient administration of doxycycline-induced Cas9 expression using a piggyBac transposon. Transient DOX administration and gRNA and donor oligonucleotides transfection	Correction of the *TAZ* mutation which is known to cause Barth syndrome	Edited cells grown on microchips that resemble myocardial tissue	Confirmed the role of the *TAZ* mutation in causing disease phenotypes such as disrupting sarcomere assembly and mitochondrial dysfunction	[214]
			–	Correction of the R222Q-SCN5A mutation	Used differentiated ventricular cardiomyocytes with heart-on-a-chip biowires	Electrically stimulated biowires facilitated the expression of the functional differences in mutated cells compared to corrected controls, including short action potentials, sarcomere disruptions, and decreased contractility	[215]

ssODN: single-stranded oligodeoxynucleotide; RPE: retinal pigment epithelium; vAFE: ventral anterior foregut endoderm.