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CRISPR STRATEGIES FOR STEM CELL ENGINEERING: A NEW FRONTIER IN MUSCULOSKELETAL REGENERATION

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Abstract

The costs and incidence of musculoskeletal injuries are rapidly increasing due to rising population age, higher prevalence of risk factors, and lack of effective long-term treatments. Regenerative medicine addresses the demand for treatments using biological cues to stimulate progenitor cells to create engineered tissues for engraftment at injury sites. However, traditional regenerative therapies are challenged by broad phenotypic changes and high risks of undesirable and systematic side-effects. In comparison to the delivery of recombinant growth factors and gene delivery approaches, clustered regularly interspaced short palindromic repeats (CRISPR) gene editing facilitates direct, specific, and tunable modification of gene expression to enable fine control over cell fate and behavior. This technology has proven to be a potent tool for the treatment of genetic diseases that impact the musculoskeletal system, such as Duchenne muscular dystrophy. However, its potential extends beyond the treatment of genetic disorders, as it also holds promise in augmenting tissue repair in patients suffering from traumatic injuries and inflammatory conditions. This review delves into the recent progress and future prospects of CRISPR-based strategies in musculoskeletal tissue engineering. Particular emphasis is placed on describing the different CRISPR modalities, delivery systems and their mechanisms of action, highlighting their potential in enhancing the repair of bone, cartilage, skeletal muscle, tendon, and ligament tissues.

Keywords: CRISPR, gene editing, stem cell biomanufacturing, regenerative medicine, osteogenesis, chondrogenesis, myogenesis.

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List of Abbreviations

AAV	adeno-associated virus
ALP	alkaline phosphatase
BCL11A	BCL11 transcription factor A
BMP9	recombinant bone morphogenetic protein 9
BV	baculovirus
CAR	chimeric antigen receptor
Cas	CRISPR-associated proteins

CRISPR	clustered regularly interspaced short palindromic repeat
CRISPRa	CRISPR activation
CRISPRi	CRISPR interference
CRISPRki	CRIPSR gene knock-in
CRISPRko	CRISPR gene knock-out
crRNA	CRISPR RNA
DANCR	differentiation antagonizing non-protein coding RNA
dCas	dead Cas
DMD	Duchene muscular dystrophy
DNMT	DNA methyltransferase
DSB	double stranded break
dsDNA	double stranded DNA
DUB	deubiquitinating enzyme
ECM	extracellular matrix
FST	follistatin
GRASLND	glycosaminoglycan regulatory associated long non-coding RNA
GRN	gene regulatory network
gRNA	guide RNAs
HDR	homology directed repair
Hes-1	hairy and enhancer of split protein 1
HP1-α	heterochromatin protein 1 alpha
IFNG	interferon gamma
IGF1	insulin like growth factor 1
IL-1	interleukin 1 complex
IL-1β	interleukin 1 beta
IL1R	interleukin 1 receptor
IL1RN	interleukin 1 receptor antagonist
IL1RAP	interleukin 1 receptor accessory protein
IL-6	interleukin 6
iPSCs	induced pluripotent stem cells
KRAB	Kruppel-associated box
lncRNA	long non-coding RNAs
MCP1	monocyte chemoattractant protein 1
miR-140	microRNA 140
MKX	mohawk
MMEJ	microhomology-mediated end joining
MMP13	matrix metallopeptidase 13
mRNA	messenger RNA
MSC	mesenchymal stem cells
MSX1	muscle segment homeobox 1
MYC	MYC proto-oncogene
MYF4	myogenic regulatory factor 4
MYF5	myogenic Factor 5
MYOD1	myogenic differentiation gene 1
MYOG	myogenin
NGF	nerve growth factor
NHEJ	non-homologous end joining
NOG	noggin
OA	osteoarthritis
OI	osteogenesis imperfecta
p65	nuclear factor kappa B subunit 3
PAX7	master transcription factor paired box protein 7
PD1	programmed death 1
PDF	platelet derived growth factor
pDNA	plasmid DNA
PEI	polyethylenimine
PPARG	peroxisome proliferator-activated receptor gamma
RNP	ribonucleic proteins
Rta	replication and transcription activator ORF50
SC	satellite cells
SCX	scleraxis
SEPT2	septin 2
SOX9	sex-determining region Y transcription factor 9
ssODN	single stranded oligodeoxynucleotide
TAK1	transforming growth factor β-activated kinase 1

TALENS	transcription activator-like effector nucleases
TET	ten-eleven translocation dioxygenases
TNF-α	tissue necrosis factor alpha
tracrRNA	trans activating CRISPR RNA
TSS	transcriptional start site
VEGF	vascular endothelial growth factor
VML	volumetric muscle loss
VP64	virion protein 64
VPR	VP64-p65-Rta
WRPW	tryptophan, arginine, proline, tryptophan
ZFN	zinc-finger nucleases
ZIF-8	zeolitic imidazolate frameworks nanoparticles

Introduction

Changes in gene expression guide the tissue regeneration process after trauma and disease. Regenerative medicine strategies targeting the musculoskeletal system have traditionally attempted to manipulate gene expression profiles by providing cells with biological and physical stimuli to enhance progenitor cell differentiation and tissue-specific extracellular matrix (ECM) production. However, the therapeutic relevance of these strategies remains limited due to low *in vivo* residence times of recombinant growth factors and the quick modification and degradation of material properties upon implantation.

Furthermore, these strategies induce broad changes in gene expression and cell behavior which reduces their specificity. In contrast, gene therapy can directly stimulate the gene expression networks responsible for stem cell regenerative capabilities. Despite the demonstrated capacity of viral and non-viral delivery gene delivery to upregulate specific tissue-associated genes, these strategies are still limited by the uncontrolled genomic integration of viral delivery or insufficient expression levels when using non-viral methods. To overcome this, synthetic biology tools offer a more precise, safer, and versatile approach for the alteration of genomic DNA and the induction of sustained long-lasting changes in gene expression.

The clustered regularly interspaced short palindromic repeat (CRISPR) gene editing system is inspired by the prokaryotic immune system which identifies, memorizes, and targets viral nucleic acids to evade infection (Jinek *et al*., 2012). The efficiency of the CRISPR associated (Cas) enzymes responsible for targeting these nucleic acids motivated its adaptation for editing genomic DNA in eukaryotic cells. Cas endonucleases add ease of design, increased specificity and efficiency, and multiplexed targeting to advance gene editing past the limitations of previous techniques such as zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENS) (Ikmi *et al*., 2014).

Early research applied CRISPR for loss of function studies in which Cas endonucleases reduce or eliminate the expression of a single gene to understand its biological role (Fan *et al*., 2022). These studies enabled the characterization of a wide range of genes and their contribution to physiological processes, disease progression, and regenerative pathways (Heckl *et al*., 2014; Malina *et al*., 2013). CRISPR has since evolved for more advanced therapeutic applications including the correction of genetic diseases such as Huntington's disease or sickle cell anemia, the prevention of antibiotic resistance through the treatment of bacterial infections, and the production of enhanced chimeric antigen receptor (CAR) T-cells for cancer therapies (Finkel *et al*., 2016; Frangoul *et al*., 2021; Su *et al*., 2016). In fact, Vertex Pharmaceuticals is poised to become the first company to receive FDA approval for a CRISPR therapy that focuses on the *ex vivo* knock-out of the B-cell lymphoma/leukemia 11A (*BCL11A*) gene in hematopoietic stem cells for treating sickle cell anemia (Kingwell, 2023). The successful approval of this therapy would serve as a significant milestone, paving the way for the application of CRISPR in a wide range of therapeutic areas including musculoskeletal regeneration.

This review will abridge the past and present of CRISPR for the regeneration of bone, cartilage, skeletal muscle, tendon, and ligament, highlighting the current applications, limitations,

and future directions of this technology from a biomedical engineering perspective.

Fundamental concepts for CRISPR gene editing

CRISPR is a biologically inspired gene editing tool that was first identified as an adaptive immune system in archaea and bacteria (Fig. 1) (Mojica *et al*., 2005). In prokaryotes, this immune mechanism is composed of multiple Cas proteins that work in tandem to copy sequences from foreign nucleic acids and store them in their own genome (Fig. 1**A**). The clustered regulatory interspaced short palindromic repeats (CRISPR) acronym is named after the identical repeat sequences that separate the copied viral DNA when it is integrated in the cell's genome at the CRISPR array (Barrangou *et al*., 2007). During viral infections, these memory sequences are translated into guide RNAs (gRNA) which then direct Cas endonucleases, such as Cas9, to degrade the viral nucleic acids and protect the bacteria (Fig. 1**A**) (Garneau *et al*., 2010). The ability to bind and cleave a unique DNA sequence without compromising the cell's genomic inspired the adaption of CRISPR as a gene editing tool in eukaryotic cells (Fig. 1**B**) (Jinek *et al*., 2012). To accomplish this, the properties and functions of Cas proteins and gRNAs have been adapted from prokaryotes to produce a highly specific and tunable gene editing tool (Jinek *et al*., 2012).

Eukaryotic CRISPR systems are classified based on the structure and phylogeny of their prokaryotic Cas endonuclease counterparts. Class II CRISPR systems are preferred for medical applications as they employ Cas proteins that function as monomers which simplifies delivery and design (Jinek *et al*., 2012). All Class II CRISPR mechanisms require two components: (1) a Cas endonuclease and (2) a suitable gRNA.

Class II Cas endonucleases

Cas endonucleases are the key facilitators in CRISPR-mediated gene editing. These proteins may be delivered to a cell in plasmid DNA (pDNA), RNA, or protein format based on the application and the delivery vehicle being used (*see Section 5. Delivery Methods*). Among all class II endonucleases, Cas9 was the first to be applied in eukaryotes and remains the most well-characterized (Jinek *et al*., 2012). Despite the popularity of Cas9, there are many class II Cas endonucleases displaying unique characteristics and cleavage mechanisms which can be tailored to specific applications. For example, while Cas9 cleaves double stranded DNA (dsDNA) at adjacent locations on each strand leaving a blunt double stranded break (DSB), Cas12a cleaves dsDNA in non-adjacent locations leaving short single stranded ends (Zetsche *et al*., 2015). Cas12a may therefore facilitate higher knock-in efficiency, especially for single nucleotide knock-ins due to the sticky end overhangs (*see Section 3. CRISPR knock-in*). Similarly, Cas13 possesses RNase activity which allows rapid and direct gene expression inhibition without the mutation risks of targeting of genomic DNA (Abudayyeh *et al*., 2017). Cas endonucleases have also been engineered to eliminate DNA cleavage activity (*see Section 4. CRISPR for Transcriptional Regulation*) or to reduce their size to ease their cellular delivery (Xu *et al*., 2021).

Guide RNA

Unique gRNAs are required to activate and direct each Cas endonuclease towards a specific sequence in the genome. gRNAs consist of two distinct segments: CRISPR RNA (crRNA) and trans activating CRISPR RNA (tracrRNA) which dictate the cleavage location and facilitate complexing with the endonuclease respectively.

A) CRISPR RNA (crRNA): The crRNA segment is inspired by the sequences that prokaryotes copy from viral DNA and integrate into their own genome for later guidance of the Cas endonucleases to specific viral DNA. The crRNA is a 20-40 bp sequence complementary to the target sequence that dictates the exact Cas cleavage location. crRNA may be re-designed for each application and target site while all other CRISPR components are kept unchanged, giving CRISPR exceptional tunability. When designing crRNA, it is important that each target sequence is immediately upstream of a protospacer adjacent motif (PAM) sequence on the genomic DNA (Jinek *et al*., 2012). The PAM is a short (3-5 bp) sequence that is used by the Cas endonuclease to verify that the target DNA is foreign before cleavage (Mojica *et al*., 2009). *Streptococcus pyogenes* Cas9 requires a 5'-NGG-3' PAM, but other isoforms use distinct PAMs, which must be

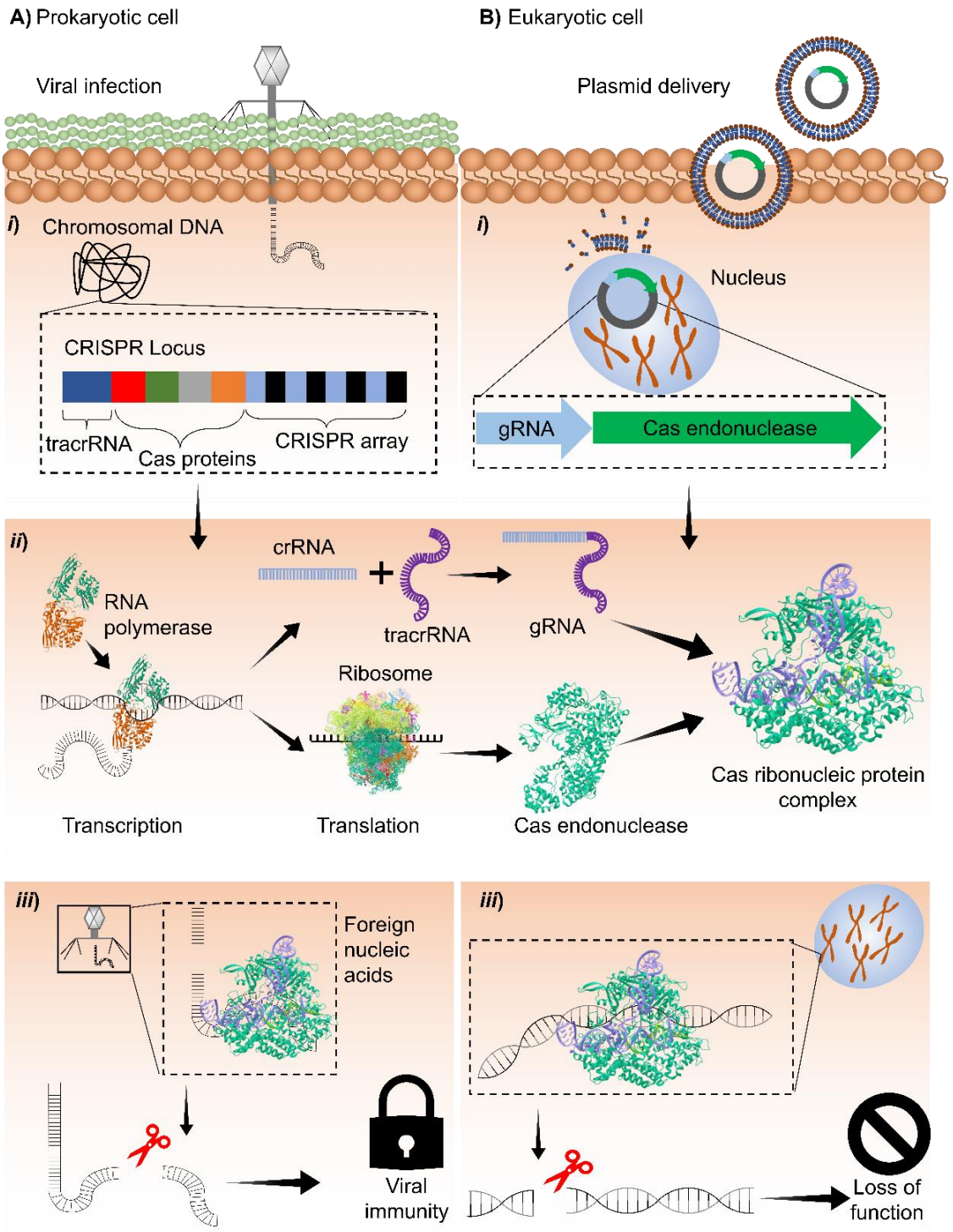

Fig. 1. CRISPR mechanisms in bacteria/archaea and their adaptation to eukaryotic cells. A) In prokaryotic cells, *i*) CRISPR associated (Cas) proteins from the CRISPR locus are deployed upon viral infection to integrate short yet specific viral DNA sequences into the CRISPR array. *ii*) During subsequent infections, these sequences are transcribed into non-coding CRISPR RNAs (crRNAs) that interact with the trans activating CRISPR RNA (tracrRNA) sequence to form the guide RNA (gRNA) sequence. gRNAs complex with Cas endonucleases to form a ribonucleic complex that subsequently *iii*) cleaves the viral DNA to grant the cell viral immunity. **B)** In eukaryotes *i*) the gRNA and Cas endonuclease must be intentionally delivered to the cell nucleus using viral or non-viral methods before *ii*) transcription of gRNAs, translation of Cas endonuclease, and ribonucleic protein (RNP) complexing. *iii*) This complex then identifies and cleaves the target sequence dictated by the gRNA resulting in an immediate loss of gene function.

considered when selecting crRNA target sequences. Off-target binding sites, self-complementarity, and guanine/cytosine content must also be considered to optimize the efficiency of gene editing and reduce off-target effects.

B) Trans activating CRISPR RNA (tracrRNA): The second component of the guide RNA is the tracrRNA, which is responsible for complexing crRNA with Cas endonucleases. This sequence exhibits a loop structure that is recognized by the endonuclease and facilitates the interaction of gRNA and the Cas protein for the formation of an RNP complex. The unique loop structure of tracrRNA presents crRNA in the correct orientation and alters the protein conformation, inducing an active endonuclease state.

C) Single guide RNA: Prokaryotic CRISPR mechanisms rely on crRNA and tracrRNA transcripts annealing together before they complex with the Cas endonuclease and direct DNA cleavage. For applications involving eukaryotic cells, the individual crRNA and tracrRNA components can be fused together to produce a single guide RNA (sgRNA), which simplifies CRISPR delivery and action.

Following delivery into the cell, the Cas endonuclease and sgRNA must first complex to form an RNP complex. This complex then enters the nucleus, identifies the genetic target sequence, and cleaves the nucleic acid. The cleavage of genomic DNA temporarily halts transcription of all downstream genes until the next transcriptional start site (TSS), causing a loss of function or knock-out of the gene. Natural eukaryotic repair mechanisms will ligate the broken DNA in an error prone method that often causes frame-shift mutations and disrupted gene function (*see Section 3. CRISPR Knock-out*). A similar system can also be used to insert a target gene at the cleavage site resulting in a gain of function or knock-in. This technique involves the co-delivery of the RNP with a donor gene sequence containing the target gene that will be inserted at the cleavage site (*see Section 3. CRISPR knock-in*). Additional techniques use a modified version of the Cas endonuclease with no cleavage capacity, dead Cas (dCas), which can be used in combination with transcriptional regulators to modulate the expression of a specific gene sequence (*see Section 4. CRISPR for Transcriptional Regulation*). Overall, CRISPR enables highly specific gene editing via the delivery of only two components and has therefore greatly advanced the precision, tunability, and range of applications of gene editing.

Direct genomic sequence modification with CRISPR

Prokaryotic Cas proteins are highly efficient endonucleases that cleave unique DNA sequences to halt gene transcription. The first examples of CRISPR's application in eukaryotes used these enzymes in their natural state to cleave genomic DNA for the inhibition of target genes or gene knock-out (CRISPRko) and the insertion of exogenous genes or gene knock-in (CRISPRki) (Fig. 2) (Liang *et al*., 2015).

CRISPR knock-out (CRISPRko)

In prokaryotes, Cas endonucleases target and cleave sequences of viral pathogens producing DSBs which cannot be repaired by the viral machinery, therefore granting viral immunity to the host (Fig. 1**A**) (Jinek *et al*., 2012). Similarly, in eukaryotic cells, this DNA cleavage causes a halt in transcription at the DSB, but the effect is limited to only the targeted gene, since RNA polymerase will begin transcription again at the next downstream TSS (Fig. 2**A**). Hence, the use of Cas endonucleases enables the silencing of specific genes without risking damage to the entire genome. In contrast to viral nucleic acids in prokaryotes, eukaryotic cells possess natural repair mechanisms for DSBs via non-homologous end joining (NHEJ). Here, the cleaved DNA ends are ligated together to re-stabilize the genome. This repair method often causes insert/deletion (indel) mutations at the site of the DSB, disrupting the target gene sequence through genetic frameshifts, which results in the production of inactive or fragmented proteins (Yuan *et al*., 2021).

CRISPRko is especially useful for loss of function studies and gene characterization. Additionally, CRISPRko can be applied to removing deleterious genes for therapeutic purposes. The main example of this application is the knock-out of the programmed death-1 (PD1) receptor in CAR T-cells to enhance T-cell proliferation and function (Su *et al*., 2016). In musculoskeletal regeneration, similar approaches

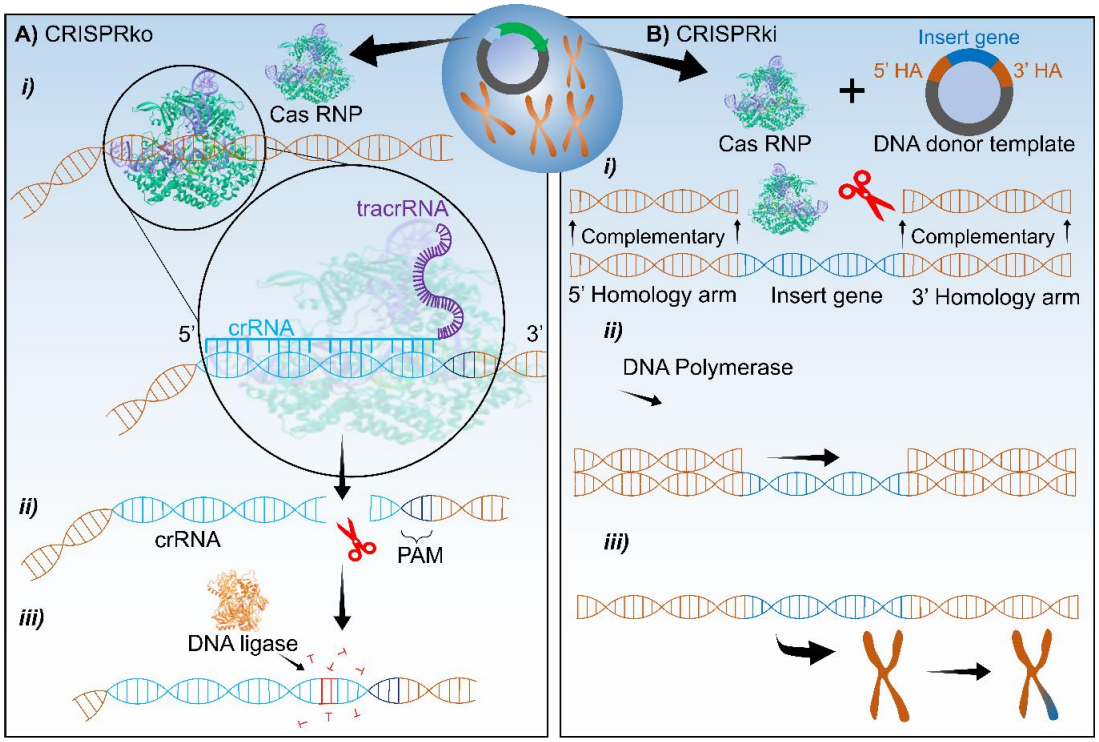

Fig. 2. Mechanisms for CRISPR knock-out (CRISPRko) and CRISPR knock-in (CRISPRki). A) Once the Cas ribonucleic protein (RNP) enters the cell nucleus, it *i*) identifies the genomic sequence complementary to the CRISPR RNA (crRNA) portion of the guide RNA (gRNA) and *ii*) induces a double stranded break (DSB) in the DNA. *iii*)The innate non-homologous end joining (NHEJ) pathway repairs the break using DNA ligase to bridge the DSB with random nucleotides from the surrounding nuclear milieu causing random indel mutations. **B)** For CRISPRki, Cas RNP delivery is supplemented with a donor DNA sequence containing the gene to be inserted flanked by homology arms complementary to the genomic DNA adjacent to the DSB. *i*) After Cas endonuclease cleavage, the homology arms of this donor sequence line up the insert gene over the DSB site so that *ii*) DNA polymerase copies the insert gene into the duplicated chromosomal DNA during cell division resulting in *iii*) sustained expression of the transgene.

have also been explored. For example, Brunger *et al*. (2017b) used CRISPR/Cas9 to knock-out genes encoding interleukin-1 (IL-1) receptor to reduce the impact of inflammatory signals on chondrocytes during osteoarthritis (OA). Although CRISPR knock-outs have laid the framework for CRISPR gene editing, its clinical application is limited by high frame shift mutation risks which can disrupt complex gene networks.

CRISPR knock-in (CRISPRki)

CRISPR can be utilized for gene knock-in or gene correction, offering a gain-of-function therapy approach. In its simplest form, natural NHEJ pathways following CRISPR DSBs can create equal and opposite indels to correct frameshift mutations at a genetic defect site (Liang *et al*., 2015). This method is applicable to the treatment of many genetic diseases such as Duchene muscular dystrophy (DMD), however the reliance on cell selection and limitations to single nucleotide knock-ins restricts its therapeutic potential (Amoasii *et al*., 2018). To overcome these limitations, CRISPR has been optimized to facilitate the knock-in of large transgenes into specific genomic locations, allowing for constitutive expression of the inserted sequence (Fig. 2**B**). CRISPRki technology is based on homology directed repair (HDR) to align a transgene over the DSB site so that the new gene can be inserted into the genome during cell division (Fig. 2**B**) (Zhang *et al*., 2017). These transgenes are delivered into the cell as plasmid DNA (pDNA), linear DNA or single stranded oligodeoxynucleotide (ssODN) donor templates

containing the gene of interest flanked by homology arms complementary to either side of the DSB site (Fig. 2**B**) (Hisano *et al.*, 2015; Yoshimi *et al.*, 2016). Successful examples of CRISPRki in regenerative medicine include the incorporation of a *COL1A1* transgene into human iPSCs for enhanced ECM production *in vitro* and the knock-in of *IL1RA* under inducible promoters to regulate inflammation throughout *in vitro* cartilage models (Jung *et al.*, 2021; Pferdehirt *et al.*, 2019).

Although such strategies have been successful, CRISPRki remains limited due to the need for donor template-DSB alignment during active DNA replication (S or G2 phase of division), making it difficult to effectively coordinate RNP and donor template delivery (Saleh-Gohari, 2004). Multiple strategies have been developed to address the low efficiency of CRISPRki including:

A) Optimizing the donor sequence: The DNA donor template can be modified to increase its stability and affinity for the target site. Strategies for this purpose include homology-mediated end joining (HMEJ), microhomology-mediated end joining (MMEJ), Tild-CRISPR, and Easi-CRISPR systems (Nakade *et al.*, 2014; Quadros *et al.*, 2017; Yao *et al.*, 2018). HMEJ employs a donor plasmid containing sgRNA target sequences adjacent to each homology arm so that Cas9 simultaneously cleaves the dsDNA donor and genomic DNA. HMEJ therefore increases the chances that the transgene will align over the DSB since the donor sequence will be liberated from its plasmid during the peak expression of the Cas endonuclease. MMEJ uses a similar strategy in which the gene for insertion is flanked with small (5-10 bp) homology arms to allow insertion during all phases of cell division. However, this methodology fails to stably integrate the donor sequence when applied to cultured cells due to reduced specificity (Nakade *et al.*, 2014). More recently, Tild-CRISPR improved knock-in efficiency 12-fold by delivering a linear dsDNA template produced *in vitro* alongside Cas9 mRNA (Yao *et al.*, 2018). Alternatively, Easi-CRISPR uses an ssDNA donor template delivered with CRISPR ribonucleic proteins (RNPs), allowing editing efficiencies up to 100 % in mouse zygotes (Quadros *et al.*, 2017). Despite the many options available for the design and optimization of donor sequences, selecting an appropriate donor template will require a detailed evaluation of the delivery format, cell type, and length of the transgene to best increase knock-in efficiency.

B) Selecting the appropriate Cas endonuclease: The type of Cas endonuclease also plays an important role in knock-in efficiency. For example, the small overhangs resultant of Cas12a cleavage can be used as sticky ends to help align donor templates. Alternatively, Cas proteins can be mutated to produce enhanced endonucleases such as Cas9 nickase which only cuts a single strand of dsDNA. Cas9 nickase eliminates off target cleavage and insertion by requiring two unique sgRNA targeting either side of the dsDNA to create the double stranded break, however this requirement causes a lower rate of DSBs and therefore a reduced insertion rate (Shen *et al.*, 2014).

While many CRISPRki strategies show advancements in editing efficiency, the overall insertion rate for CRISPRki remains low, making its incorporation into regenerative medicine challenging, especially in those applications needing a high percentage of edited cells to elicit a therapeutic response.

CRISPR for transcriptional regulation

CRISPR also allows for the modulation of gene expression levels without changing the genomic sequence. To accomplish this, CRISPR is reconfigured to act as a precise and tunable delivery system for transcriptional regulators which upregulate (CRISPR activation, CRISPRa) or downregulate (CRISPR inhibition, CRISPRi) the expression of target genes (Fig. 3). In such systems, the Cas endonucleases are modified resulting in an inactive or dCas mutant void of nuclease activity. Hence, dCas effectors retain their specificity for the target locations dictated by their gRNA, but no longer cleave the target sequence (Chavez *et al.*, 2015). dCas proteins may then be fused to transcriptional regulators allowing their delivery to specific genomic sequences and the regulation of gene expression (Fig. 3). This methodology is especially relevant for regenerative medicine since the regulation of endogenous gene expression is a simpler and safer strategy than gene knock-out or knock-in for controlling cell function (Chakraborty *et al.*, 2014).

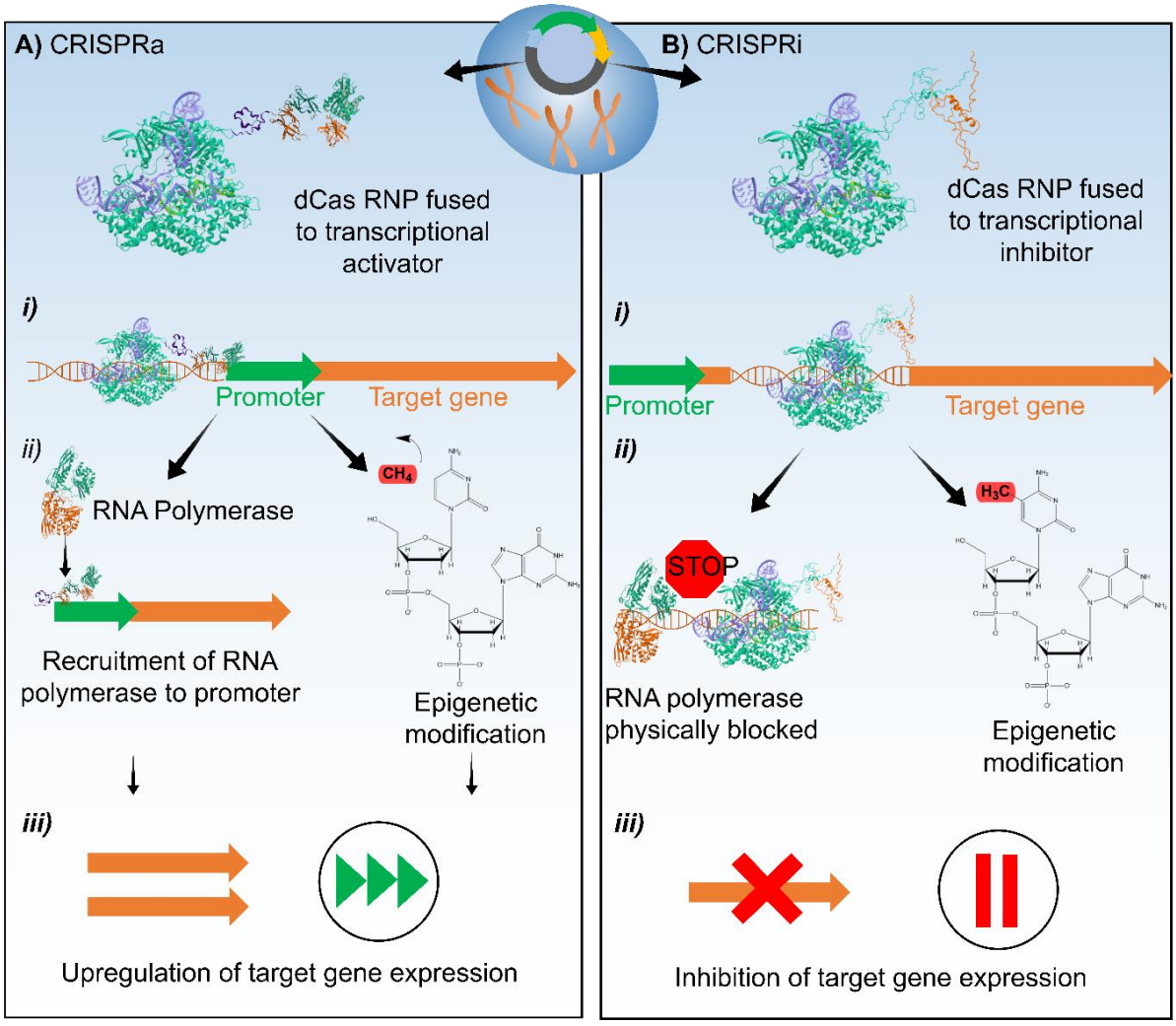

Fig. 3. Mechanisms for CRISPR activation (CRISPRa) and CRISPR inhibition (CRISPRi). A) dead CRISPR associated (dCas) endonucleases fused with transcriptional activators are delivered to the nucleus of the cell where they *i*) bind to the target sequence 400-50 bp upstream of the promoter sequence. *ii*) The presence of transcriptional activators recruits RNA polymerase to the promoter region or directly modifies the DNA through mechanisms such as demethylation to *iii)* activate gene expression. **B)** dCas endonucleases fused with epigenetic modifiers for gene repression are delivered to the nucleus of the cell where they *i*) bind to the target sequence 50-100 bp downstream of the promoter sequence. *ii*) The presence of the dCas ribonucleic protein (RNP) physically blocks RNA polymerase from continuing transcription and the transcriptional repressors directly modify the DNA through mechanisms such as methylation to *iii)* inhibit gene expression.

CRISPR activation (CRISPRa)

CRISPRa is used to upregulate target gene expression by delivering transcription factor activation domains to the promoter region of a gene (Fig. 3**A**). In CRISPRa, sgRNA direct the binding of dCas proteins equipped with activation domains 400-50 bp upstream of the target gene's promoter region (Perez-Pinera *et al.*, 2013). The activation domains either directly interact with the DNA to increase expression levels or recruit RNA polymerase to upregulate gene expression. CRISPRa allows fine control over expression levels since the delivery of multiple sgRNA for the same target gene recruits more transcriptional activators, resulting in a cumulative effect over gene expression (Chavez *et al.*, 2015). CRISPRa applications in regenerative medicine include the activation of myogenic differentiation gene 1 (*MYOD1)* to induce the myogenic differentiation of murine fibroblasts or the upregulation of bone morphogenetic protein 9 (*BMP9)* to improve muscle regeneration in

murine MSCs (Chakraborty *et al*., 2014; Freitas *et al*., 2021). Furthermore, CRISPRa can be applied in a multiplexed approach to activate multiple genes simultaneously and recapitulate gene expression networks (Perez-Pinera *et al*., 2013). Efforts have been focused on maximizing gene activation levels in CRISPRa by exploring various activator domains and improving fusion mechanisms between the activator and dCas protein.

A) Transcriptional activators for CRISPRa: Initially, transcriptional activators such as virion protein 64 (VP64), nuclear factor kappa B subunit 3 (p65), or replication and transcription activator ORF50 (Rta) were directly fused to the terminus end of the dCas protein (Chavez *et al*., 2015; Gilbert *et al*., 2013; Perez-Pinera *et al*., 2013). Although, individual delivery of these activators showed mild efficacy, Chavez *et al.* (2015) found that the combination of all three to form a VP64-p65-Rta (VPR) domain increased expression up to 20-fold. An alternative approach is the use of domains responsible for epigenetic modification of DNA through methylation or acetylation. Such regulators include p300, ten-eleven translocation dioxygenases (TET) and DNA methyltransferase, which can be fused to dCas proteins to chemically modify DNA and activate expression comparably with dCas-VPR (Amabile *et al*., 2016; Hathaway *et al*., 2012; Hilton *et al*., 2015). The genetic makeup of target genes and cellular environment should be considered during CRISPRa to select the transcriptional regulator that can best reach and interact with the target sequence.

B) Activation domain fusion to dCas protein: To further improve CRISPRa, the delivery of activation domains can be optimized. Different systems such as Scaffold, Casilio, and Cas9-SAM attach transcriptional activators to the guide RNA loops left exposed after RNP formation to better orient the activators towards the target DNA (Cheng *et al*., 2016; Tanenbaum *et al*., 2014; Zalatan *et al*., 2015). In the Sun-Tag system, dCas proteins are modified to present peptide sequences that selectively interact with modified activation domains. This enables the delivery of different activators tailored to the specific requirements of each target gene. These approaches have shown up to 5-fold greater activation using CRISPRa, however post translational assembly and added complexing steps hinders their use in regenerative medicine (Konermann *et al*., 2015).

CRISPR interference (CRISPRi)

dCas proteins are also used to suppress gene expression in CRISPRi (Fig. 3**B**). In comparison to CRISPRko, CRISPRi has no mutation risks and enables multiplexed gene inhibition by incorporating multiple gRNAs (Gilbert *et al*., 2014; Qi *et al*., 2013). The targeted binding of dCas9 50-100 bp downstream of the TSS physically blocks RNA polymerase during transcription, resulting in a 2-fold inhibition of gene expression (Gilbert *et al*., 2013; Qi *et al*., 2013). To improve inhibition levels, epigenetic inspired chromatin-modifying domains such as the Kruppel-associated box (KRAB) domain, the chromatin shadow (CS) domain of heterochromatin protein 1 alpha (HP1-α), and the tryptophan, arginine, proline, tryptophan (WRPW) domain of hairy and enhancer of split protein 1 (Hes-1), can be bound to dCas9 (Gilbert *et al*., 2013). More recent methods have improved gene repression by incorporating DNA methyltransferase (DNMT) either by N-terminal fusion to dCas or via the SunTag system. However, dCas-DMNT systems often methylate entire genomic regions resulting in the inhibition of multiple genes and therefore causing off-target effects (Amabile *et al*., 2016).

CRISPRi has been explored in musculoskeletal regeneration for inhibition of adipogenic genes to promote cartilage formation and subsequent endochondral ossification in adipose derived stem cells (Truong *et al*., 2022). The main limitation to CRISPRi is that many genes share the same TSS, therefore, inhibiting one gene could inhibit multiple downstream genes (Gilbert *et al*., 2013). Further research is needed to improve gene specificity and inhibition levels to increase CRISPRi's therapeutic relevance.

CRISPR delivery

CRISPR delivery format

The first consideration when aiming to deliver CRISPR machinery is the format of the different CRISPR components (Table 1). Cas endonucleases can be delivered as pDNA, messenger RNA (mRNA) and protein. The initial application of CRISPR technology for editing

mammalian cells involved the use of pDNA to achieve Cas9 protein overexpression (Cong *et al.*, 2013). This approach provided a straightforward and cost-effective solution. However, pDNA is limited by slow editing action (pDNA must be transcribed and translated for protein synthesis), increased off-target effects due to prolonged Cas9 production, and risk of insertional mutagenesis (Merkle *et al.*, 2015; Ono *et al.*, 2019). Compared to pDNA delivery, mRNA enables faster endonuclease expression through direct cytoplasmic translation. Moreover, mRNA eliminates the risks of insertional mutagenesis and minimizes off-target editing due to its short half-life (Shen *et al.*, 2014). Finally, Cas9 can be delivered as a protein, showing similar advantages to mRNA, such as reduced off-target effects and no insertional mutagenesis risk. Despite these advantages, the Cas9 protein is positively charged (net charge = +22) and has a relatively high size (~160 kDa), which complicates its delivery through the cell membrane (Liu *et al.*, 2017). The Cas9 protein can also be complexed to the gRNA prior to delivery, forming a negatively charged RNP complex. This facilitates its delivery through positively charged liposomes and cationic polymers (Zuris *et al.*, 2015). Different studies have systematically compared which format offers higher editing efficiency and less off-target effects. Kouranova *et al.* (2016) compared the delivery of CRISPR components as DNA, RNA, and protein, reporting highest nuclease activity when Cas9 was delivered as a protein or when stably expressed through viral transduction. Despite these previous observations, the CRISPR delivery format should be optimized for each individual application and cell type.

Table 1. Comparison between the different Cas9 delivery formats.

Cas format	Editing speed	Stability	Off-target effects	Insertional risk	Ease of delivery
DNA	Low (transcription and translation required)	Sustained expression	Yes	Yes	Moderate (nuclear delivery needed)
RNA	Moderate (translation required)	Quickly degraded	Limited	None	Easy (delivery to cell cytoplasm)
Protein	Quick	Quickly degraded	Limited	None	Moderate (limited by protein size and charge)

CRISPR delivery methods

In addition to CRISPR delivery format, successful gene editing also depends on the delivery strategy. This can be achieved through viral and non-viral delivery systems.

A) Viral Delivery: Viral delivery exhibits more robust CRISPR gene editing than non-viral delivery due to the stable expression of CRISPR components. However, these vectors face critical barriers such as patient safety due to random genomic integration and immunogenicity. Among various viral gene delivery systems, adeno-associated viruses (AAVs) are rapidly advancing towards clinical trials. This is primarily attributed to their low immunogenicity and their non-integrating nature. However, recent reports focusing on AAV delivery of CRISPR/Cas9 have identified random genomic integrations of AVV elements into Cas9-induced DSBs (Hanlon *et al.*, 2019; Nelson *et al.*, 2019). Additionally, Chew *et al.* (2016) reported immunogenicity of AAV-based delivery of CRISPR/Cas9 and destabilization of the hosts immune system after administration. Similar instances have been described when using alternative viral vectors such as adenoviruses and lentiviruses for CRISPR delivery (Lee *et al.*, 2021; Manjón *et al.*, 2022; Wang *et al.*, 2015). Additional hurdles are the restricted packaging capacity, and difficulty of large-scale production. A single AAV is not sufficient for

packing all the necessary CRISPR components since the Cas9 sequence has a size of ~4.2 kb in comparison to the AAVs packaging capacity of ~4.5 kb, therefore dual vector systems are required, increasing production costs (Mefferd *et al*., 2015). Recent studies have identified the insect baculovirus (BV) as a promising alternative for CRISPR delivery, due to the lack of genomic insertion, larger packaging capacity, and no pathogenicity to humans (Hindriksen *et al*., 2017). Despite effective gene transfer and gene editing, constitutive Cas9 overexpression remains as a further limitation of all viral vectors, as the continuous presence of the Cas nuclease might lead to off-target gene cleavage and unwanted side effects (Breton *et al*., 2020).

B) Non-Viral Delivery: Non-viral delivery is based on the use of chemical compounds and/or physical processes for gene transfer. These techniques demonstrate improved safety profile and ease of production in comparison to their viral counterparts. Additionally, the transient action of Cas endonucleases when delivered through these methods reduces the risks of off-target effects and immune reactions. Physical methods are based on the transient opening of the cell membrane through microinjection, electroporation and sonoporation. Despite physical techniques are widely used for CRISPR delivery to a variety of targets such as embryos (Alghadban *et al*., 2020), induced pluripotent stem cells (iPSCs) (Liang *et al*., 2015) and organoids (Hendriks *et al*., 2021), they are limited to *ex vivo* cell manipulation, require special equipment, and are labor intensive. To address these challenges, various groups have modified these techniques for *in vivo* delivery (Shinmyo *et al*., 2016). However, obstacles such as tissue disruption and immune activation still limit their application primarily to *in vitro* manipulation. In contrast, nanoparticle-based transfection allows for *in vitro* and *in vivo* delivery of CRISPR machinery. Between these, lipid-based nanoparticle systems are the preferred method for CRISPR delivery in different forms such as pDNA, mRNA and RNP (Cong *et al*., 2013; Li *et al*., 2019; Qiu *et al*., 2021). Similarly, cationic polymers such as polyethylenimine (PEI) also form nanoparticle complexes with CRISPR components due to electrostatic interactions, allowing for their cellular delivery (Ryu *et al*., 2018). However, widely used lipid-based and cationic polymer systems are limited by high cytotoxicity (Chen *et al*., 2019), long-term storage instability (Chen *et al*., 2019; Payton *et al*., 2014), and immunogenicity (Guo *et al*., 2019; Wei *et al*., 2015). Additionally, cationic polymers can also disrupt cell cytoskeleton and stem cell differentiation capacity, which negatively impacts their use in tissue regeneration (Gonzalez-Fernandez *et al*., 2017). Alternative approaches such as gold nanoparticles, cell penetrating peptides and DNA origami nanostructures have been investigated to offer enhanced editing efficiencies and reduced cytotoxicity (Lee *et al*., 2019; Lin-Shiao *et al*., 2022; Mbugua *et al*., 2019).

CRISPR for bone regeneration

Bone is a highly vascularized tissue with self-healing capacity. However, critically sized defects (non-unions) result in impaired wound healing and prolonged injury (Ekegren *et al*., 2018). Non-union injuries occur in approximately 5-10 % of long bone fractures (Ekegren et al., 2018). While total cost per non-union can vary due to the incidents of complications and second surgeries, they often exceed $25,000 (Ekegren *et al*., 2018). These cases are clinically addressed using autologous bone grafts, however the tissue available for grafting is limited and donor site morbidity can develop (Pape *et al*., 2010). An alternative option is the use of biomaterials in combination with the delivery of recombinant BMP-2. However, this approach requires the delivery of supraphysiological levels of the growth factor due to quick clearance and degradation at the injury site, which can lead to ectopic bone formation, systemic and local toxicity, and carcinogenesis (Carragee *et al*., 2011). CRISPR presents an alternative to recombinant proteins and traditional gene therapy approaches with advanced precision and reduction of off-target effects. Although most CRISPR applications within bone tissue engineering are restricted to the correction of genetic diseases such as osteogenesis imperfecta (OI) (Jung *et al*., 2021), CRISPR has also been used for identifying genetic targets of bone disease and increasing the bone-forming potential of progenitor cells (Table 2).

The initial application of CRISPR technology for bone repair focused on the treatment of

different genetic disorders affecting bone formation and repair. The most representative of these is OI, a rare disease that affects 1 in 25,000 births and results in brittle bones and decreased bone mass (Van Dijk and Sillence, 2014). OI is caused by a mutation in the *COL1A1*/*COL1A2* genes, which encode for the procollagen alpha chain and determine type I collagen structure. Jung *et al.* (2021) applied CRISPRki to correct the *COL1A1* gene in patient-derived iPSCs *in vitro*. These cells recovered the ability to form collagen fibrils and promote bone formation (Jung *et al*., 2021). Alternatively, rare forms of OI can result from a mutation outside of the *COL1A* genes. CRISPRki has been used to produce animal models with rare OI mutations, such as type V OI, and study disease progression (Rauch *et al*., 2018).

Nevertheless, CRISPR is not solely confined to the correction of genetic disorders; it can also be applied to augment bone repair processes through activation and inhibition of key osteogenic targets. These include the overexpression of growth factor gene families, such as bone morphogenetic proteins (*BMP)*, vascular endothelial growth factors (*VEGF*) and platelet derived growth factors (*PDF*). Freitas *et al*. (2021) explored CRISPRa to overexpress *BMP9* in mesenchymal stem cells (MSCs), resulting in an increased alkaline phosphatase (ALP) activity and mineralization. Similar strategies have been applied to *BMP4* activation (Choi *et al*., 2020). One of the key advantages of CRISPRa is its potential to simultaneously target multiple genes involved in bone healing (Chen *et al*., 2022; Hsu *et al*., 2020a). Chen *et al*. (2022) co-activated *VEGFA* and *TGFB1* using CRISPRa to increase both, vascularization, and osteogenesis, in critical bone defects. Similarly, *WNT10* and *FOXC2* have been targeted simultaneously via CRISPRa for increasing osteogenic differentiation in BMSCs (Hsu *et al*., 2020a). In contrast to CRISPRa, CRISPRi can silence genes that hinder osteogenesis, as demonstrated by Hsu *et al*. (2020b), who utilized CRISPRi to suppress noggin (*NOG*) gene expression, an inhibitor of *BMP2*, to enhance osteogenesis in adipose-derived MSCs.

A recent application of CRISPR in bone regeneration is genetic marker screening for interrogating genetic regulators of MSC osteogenesis *in vitro* and *in vivo* (Wu *et al*., 2018). Kaushal *et al.* (2022) employed CRISPRko to screen deubiquitinating enzymes (DUBs) and their effect on muscle segment homeobox 1 (*MSX1*) protein levels, a transcription factor required for normal development of various tissues including osteogenesis in human MSCs.

CRISPR for cartilage regeneration

Cartilage is a connective tissue that has poor intrinsic repair due to its avascular and aneural structure with low cell density (Ciamillo *et al*., 2023). Consequentially this tissue is vulnerable to degradation due to injury or disease, such as OA which adversely impacts over 10 % of adults aged 60 years or older (Allen *et al*., 2022). This disease is associated with increased inflammation and overexpression of pro-inflammatory cytokines such as interleukin-6 (IL-6), tissue necrosis factor alpha (TNF-α) and interleukin-1 beta (IL-1β). Clinical treatments including administration of anti-inflammatory drugs, surgery, and physical therapy, vary in their therapeutic efficacy. For instance, while anti-cytokine medication can alleviate pain associated with inflammation, it does not promote cartilage regeneration and requires high doses, which can increase the patient's susceptibility to additional infections. CRISPR has been investigated as an alternative therapeutic method to identify new genetic targets associated with OA, decrease cartilage degradation, and enhance chondrogenesis (Table 3).

CRISPRko offers an efficient approach to screen genetic targets involved in cartilage degeneration in OA, replacing traditional siRNA or shRNA-mediated knock-down methods by providing a longer-lasting genome editing effect (Chaudhry *et al*., 2022). Using primary human chondrocytes, Chaudhry *et al.* (2022) found microRNA 140 (miR-140) knock-out decreased gene expression of septin 2 (*SEPT2*), *BMP2*, and *FGF2*, as well as novel target agrin, all relevant in OA progression. Similarly, CRISPRko was used to demonstrate the key molecular role of hyaluronan in aggrecan retention during OA (Huang *et al*., 2016).

CRISPRko has also been used to target several genes upregulated in OA disease progression such as nerve growth factor (*NGF*), matrix metallopeptidase 13 (*MMP13*), and *IL1B* (Zhao *et al*., 2019). Similarly, CRISPRko of IL1 receptor (*IL1R*) and transforming growth factor β-

Table 2. CRISPR for bone tissue regeneration.

Application	Technique	Gene target	Outcome	Delivery method	*In vitro/in vivo*	Model	Reference
Correction of genetic disease	CRISPRki	*COL1A1*	Correction of *COL1A1* mutation for OI	Electroporation	*In vitro*	Human iPSCs	(Jung *et al*., 2021)
	CRIPSRko	*IFITM5*	Introduction of *IFITM5* mutation for type V OI model	Microinjection	*In vivo*	CD-1 mouse embryos	(Rauch *et al*., 2018)
Increasing bone repair	CRISPRa	*BMP9*	Overexpression of *BMP9* improved osteogenic differentiation of iMSCs and bone formation *in vivo*	Lentivirus	*In vitro/in vivo*	Immortalized Murine MSCs	(Freitas *et al*., 2021)
	CRISPRa	*BMP4*	Overexpression of *BMP4* improved osteogenic differentiation of UC-MSCs	Lipofectamine, electroporation	*In vitro*	Umbilical cord human MSCs	(Choi *et al*., 2020)
	CRISPRa	*VEGFA, TGFB1*	Overexpression of *VEGFA* and *TGFB1* improved osteogenesis and clavarial bone healing	Cationic copolymer polyaspartate	*In vitro/in vivo*	Pre-osteoblast MC3T3-E1 cells	(Chen *et al*., 2022)
	CRISPRa	*WNT10B, FOXC2*	Overexpression of *WNT10B* and *FOXC2* improved osteogenic differentiat ion and clavarial bone healing	Baculovirus	*In vitro/in vivo*	Murine BMSCs	(Hsu *et al*., 2020a)
	CRISPRi	*NOG*	Inhibition of *NOG* contributed to improved osteogenic differentiat ion of ASCs and clavarial bone healing	Baculovirus	*In vitro/in vivo*	Human ASCs	(Hsu *et al*., 2020b)
Genetic screening	CRISPRko	*MSX1*	Ubiquitin-specific protease 11 (*USP11*) is a novel protein regulator of *MSX1* and osteogenic differentiation	Lentivirus	*In vitro*	Human MSCs	(Kaush al *et al*., 2022)

activated kinase 1 (*TAK1*) have been explored for increasing chondrocyte survival under inflammatory conditions (Bonato *et al*., 2023; Brunger *et al*., 2017b). CRIPSRi and CRISPRki have also both been used to reduce OA-induced inflammation for the suppression of inflammatory cytokine receptors and the overexpression of IL1R antagonist (*IL1RN*) respectively (Farhang *et al*., 2017). Pferdehirt *et al.* (2019) used CRIPSRki to introduce *IL1RN* into the cell's genome using several strategies: both knock-in of *IL1RN* within an inflammation responsive locus and in a constitutively expressed site, as well as knock-in an inflammation responsive promoter upstream of *IL1RA*.

In addition to modulating OA effects, CRISPRa and CRIPSRi have been explored for directly enhancing chondrogenic differentiation of stem and progenitor cells. Several studies have targeted the CRISPRa-mediated activation of sex-determining region Y transcription factor 9 (*SOX9*), a key chondrogenic transcription factor (Truong *et al*., 2019; Truong *et al*., 2022). Truong *et al.* (2019) used CRISPRa and CRISPRi simultaneously to activate *SOX9* and inhibit peroxisome proliferator-activated receptor gamma (*PPARG*), a proadipogenic transcription factor, resulting in enhanced MSC chondrogenesis. Similarly, CRISPRa has been employed to simultaneously activate the expression of the SOX trio (*SOX9*, *SOX5*, and *SOX6*), leveraging CRISPRa's multiplex gene activation capabilities (Truong *et al*., 2022). CRISPR has been used to identify novel targets for chondrogenesis such as long non-coding RNAs (lncRNA).

Huynh *et al*. (2020) previously identified that glycosaminoglycan regulatory associated long non-coding RNA (*GRASLND*) as a target for enhanced MSC chondrogenesis and used CRISPRa to investigate its impact. A similar approach was also used to activate the expression of differentiation antagonizing non-protein coding RNA (*DANCR*), to demonstrate the therapeutic potential of lncRNA in cartilage regeneration (Nguyen *et al*., 2021).

CRISPR for muscle regeneration

Muscle is composed of myocytes arranged in axially aligned fibers which bundle together to create a striated pattern allowing nerve and blood vessel penetration. This vascularized and innervated nature of muscle facilitates self-healing; however, its repair capacity is compromised during trauma and surgery-induced volumetric muscle loss (VML), and degenerative disorders. Current clinical treatments such as autologous grafts fail to fully restore muscle function and risk damage to other muscles (Quarta *et al*., 2017). CRISPR gene editing could transform the treatment of muscle injuries by enabling precise and multiplexed control over myogenic gene expression and immune modulation to correct genetic diseases and direct muscle regeneration (Table 4).

CRISPR therapies for muscle regeneration have primarily been applied to the treatment of genetic diseases such as DMD, a hereditary disease caused by mutations in the dystrophin gene which is present in one in every 5,000 births. In fact, CRISPRko successfully corrected the dystrophin mutation in a canine model of this disease resulting in enhanced muscle function and regeneration (Amoasii *et al*., 2018; Bengtsson *et al*., 2017). Similarly, CRISPRki and CRISPRa have been used to insert or activate genes such as follistatin (*FST*) or laminin to enhance muscle regeneration in patients suffering from diverse genetic and degenerative diseases (Li *et al*., 2021; Perrin *et al*., 2017). Alternatively, the elimination or downregulation of mutated or atrophy-associated sequences has been accomplished using CRISPRko and CRISPRi (Himeda *et al*., 2016; Ikeda *et al*., 2020; Li *et al*., 2020).

In addition to genetic disease correction, CRISPR has also been used to enhance myogenesis and direct regeneration in traumatic muscle injuries. Most of these therapies focus on the CRISPR-mediated modification of satellite cells (SCs), which are the key progenitor cell type for muscle regeneration after injury. The genetic pathways involved in SC differentiation have inspired CRISPRa strategies aiming to upregulate the expression of various transcription factors to direct SC reprograming to myocytes. These include myogenic Factor 5 (*MYF5*), MYC proto-oncogene (*MYC*), *MYOD1*, myogenin (*MYOG*), myogenic regulatory factor 4 (*MRF4*), and the master transcription factor paired box protein 7 (*PAX7*) (He *et al*., 2021; Kabadi *et al*., 2015; Kwon *et al*., 2020). For example, Chakraborty *et al*. (2014)

Table 3. CRISPR for Cartilage regeneration.

Application	Technique	Target	Outcome	Delivery method	*In vivo/in vitro*	Model	Reference
Genetic screening	CRISPRko	miR-140	miR-140 expression is reduced during cartilage injury and effects expression of multiple arthritic gene targets	Lipofectamine	*In vitro*	Human OA chondrocytes	(Chaudhry *et al.*, 2022)
	CRIPSRko	Hyaluronan	*HAS2* knockout RCS-reduced assembly of hyaluronan and aggrecan retention	Lipofectamine	*In vitro*	Rat chondrosarcoma cell line (RCS-o)	(Huang *et al.*, 2016)
Reduction of OA inflammation	CRISPRko	*NGF*, *IL1B* or *MMP13*	Reduced expression of *NGF*, *IL1B*, and *MMP13* for reduced pain and structural damage in osteoarthritic murine joint	Adeno-associated virus	*In vivo*	Mouse BMSCs	(Zhao *et al.*, 2019)
	CRISPRko	*IL1R*	Chondrocytes differentia ted from iPSCs with knockout *IL1R* were resistant to IL1-induced degradation	Lipofectamine	*In vitro*	Murine iPSCs	(Brunger *et al.*, 2017b)
	CRISPRko	*TAK1*	TAK1 knockout chondrocytes improved integration into cartilage under inflammatory stimuli	Electroporation	*In vitro/in vivo*	Polydactyl human chondrocytes	(Bonato *et al.*, 2023)
	CRISPRi	*IL1R* and *TNFR1*	*IL1R* and *TNFR* inhibition downregulate NF-κB inflammatory signaling	Lentivirus	*In vitro*	Human ADSCs	(Farhang *et al.*, 2017)
	CRISPRki	*IL1RN*	Edited iPSCs respond to IL-1 and increase *IL1RN*	Lentivirus	*In vitro*	Murine iPSCs	(Pferdehirt *et al.*, 2019)
Increasing chondrogenic differentiation	CRISPRi/a	*SOX9*, *PPARG*	Activation of *SOX9* and inhibition of *PPARG* improved chondrogenesis and reduced adipogenesis	Baculovirus	*In vitro/in vivo*	Rat ADCs and rat BMSCs	(Truong *et al.*, 2019)
	CRISPRi/a	*SOX9*, *SOX5*, *SOX6*, *CEBPA*, *PPARG*	Activation of Sox genes and inhibition of *CEBPA* and *PPARG* improved chondrogenesis and clavarial bone defect in osteoporotic rats	Baculovirus	*In vitro /in vivo*	Rat ASCs and rat BMSCs	(Truong *et al.*, 2022)
	CRISPRa	*GRASLND*	Activation of *GRASLND* improved chondrogenesis	Lentivirus	*In vitro*	Human MSCs and human ASCs	(Huynh *et al.*, 2020)
	CRISPRa	*DANCR*	Activation of *DANCR* improved chondrogenesis and clavarial bone healing	Baculovirus	*In vitro/in vivo*	Rat ASCs and rat BMSCs	(Nguyen *et al.*, 2021)

Table 4. CRISPR for muscle regeneration.

Application	Technique	Target	Outcome	Delivery method	*In vitro/in vivo*	Model	Reference
Treatment of muscular dystrophy	CRISPRa	Laminin Subunit Alpha 1 Chain (*LAMA1*)	Strengthen Cell-ECM interactions to treat Duchene Muscular Dystrophy	Lipofectamine 2000	*In vitro/in vivo*	C2C12 murine myoblasts	(Perrin *et al.*, 2017)
	CRIPSRki	*FST*	Antagonized myostatin for upregulated muscle growth during muscular dystrophy	Electroporation	*In vitro*	Porcine fetal fibroblasts	(Li *et al.*, 2021)
Prevention of muscle atrophy	CRISPRko	miR-29b	Increased Exercise capacity and prevented muscle atrophy induced by angiotensin II	Lentivirus/adeno-associated virus 8 and local injection	*In vivo*	Murine C2C12 skeletal myoblasts, human 293T embryonic kidney cells	(Li *et al.*, 2020)
	CRISPRa	*IGF1*	Upregulation of entire IGF1 family for enhanced myotube differentiation and reduced muscle atrophy	Lentivirus	*In vitro*	Human and mouse skeletal myoblasts	(Roberston *et al.*, 2020)
Myogenic reprogramming	CRISPRko	*MYOD1, MYC*	Created a mouse model for Tamoxifen induced expression of Cas9 to evaluate impact of satellite cells on muscle regeneration	Adeno-associated Virus 9	*In vivo*	Mouse C2C12 myoblasts (CRL-1772) and SCs, human HEK2 93FT	(He *et al.*, 2021)
	CRISPRa	*PAX7*	Increased dystophin + myofibers and progenitor cell engraftment in immunodeficient mouse model	Lentivirus	*In vitro/in vivo*	Human iPSCs	(Kwon *et al.*, 2020)
	CRISPRa	*MYOD1*	Reprogrammed mouse embryonic fibroblasts to skeletal myocytes	Lentivirus	*In vitro*	Murine Embryonic Fibroblasts	(Chakraborty *et al.*, 2014)

used CRISPRa to activate the *MYOD1* transcription factor in murine fibroblasts resulting in increased levels of myogenic markers and muscle twitch responses to electrical signals. Additionally, CRISPRa has been applied to the overexpression of growth factors such as insulin like growth factor 1 (*IGF1*) to prevent steroid-induced atrophy and increase myotubule diameters *in vitro* (Roberston *et al*., 2020). These examples show the promise of using CRISPRa to activate endogenous genes for controlled cell lineage commitment and subsequent tissue formation without recombinant growth factor supplementation.

CRISPR for tendon/ligament regeneration

Tendons and ligaments are fibrous tissues primarily composed of type 1 collagen which give stability to muscle-bone and bone-bone interfaces (Tao *et al*., 2015). Both tendons and ligaments have limited healing capacity due to their lack of vascularization and low cellularity (Tao *et al*., 2015). Surgical repair and grafting treatments for tendons/ligaments are limited by donor-site morbidity, sourcing difficulties, or immune rejection; consequently, postoperative rotator cuff retears occur in 11-94 % of surgeries based on the original tear size (Cheung *et al*., 2010).

To the author's knowledge, CRISPR techniques are yet to be employed in regenerative medicine approached for tendons or ligament, presenting an opportunity to bolster the field towards clinically translatable therapies. CRISPRa should be considered to improve upon pre-existing gene therapy options and the activation of numerous growth factors encoding genes including *FGF*, *VEGF*, *PDGF*, and *TGF* for cell reprogramming to tenocytes (Chen *et al*., 2012; Tao *et al*., 2015; Zarychta-Wiśniewska *et al*., 2017) or insulin like growth factor (*IGF*), *FGF*, *PDGF*, or *TGF* for cell reprogramming to ligament fibroblasts (Haddad-Weber *et al*., 2010; Leong *et al*., 2014; Li *et al*., 2007; Provenzano *et al*., 2007; STEINERT *et al*., 2008). Additionally, the activation of the expression of transcription factors could stimulate lineage commitment, specifically scleraxis (*SCX*) and mohawk (*MKX*) (Chen *et al*., 2012; Liu *et al*., 2015). CRISPRi is also applicable to fighting immune response during tendon/ligament inflammation, autologous graft transplantation, and scaffold implantation. Specifically, interferon gamma (*IFNG*) upregulation has been correlated to damaged tendons making it a prime target for inhibition (Russo *et al*., 2022). CRISPR presents an opportunity to enhance tendon/ligament regenerative medicine therapies by adding tunability and specificity to the manipulation of tenocyte and ligament fibroblast behavior.

Future directions

Biomaterial-based delivery of CRISPR components

In vivo CRISPR delivery to adult organisms is currently limited by the lack of an effective delivery method due to rapid clearance and off-target effects (Lee *et al*., 2017). CRISPR functionalized biomaterial scaffolds present an encouraging solution for the stable and localized delivery of CRISPR machinery and *in situ* gene editing within target tissues. As such, Ho *et al*. (2021) functionalized an ECM coated hydrogel with Cas9 RNP-lipid nanoparticles to knock-out the interleukin 1 receptor accessory protein (*IL1RAP*) and inhibit pro-inflammatory pathways to slow leukemia progression. Although the scaffold enabled sustained delivery of CRISPR machinery and effective editing, the system lacked spatial definition and control over release kinetics during gene editing (Ho *et al*., 2021). Future work should develop robust, specific, and tunable biomaterial scaffolds for precise spatial and temporal control over CRISPR release kinetics to facilitate biologically relevant gene editing profiles. Furthermore, biomaterial-based delivery of CRISPR enables the recruitment of specific cell types through the co-delivery of chemo attractants and integrin-specific peptides. For example, monocyte chemoattractant protein-1 (MCP-1) or the TP H2009.1 peptide may be used to recruit macrophages or lung cancer cells respectively for the targeted editing of inflammatory or diseased cell types (Brunger *et al*., 2017b; Elayadi *et al*., 2007). This strategy will increase the specificity of CRISPR action and reduce side-effects in off-target cell populations (Greiner *et al*., 2014).

Novel nanoparticle-based delivery of CRISPR components

As described previously, non-viral nanoparticles can be used for *in vivo* delivery of CRISPR

components, however they are limited by low stability, high cytotoxicity, and low transfection efficiency. Cell-penetrating peptides are bioinspired amino acid chains capable of transfecting cells with low cytotoxicity since they are cleared rapidly after delivering their cargo. The most recent report demonstrating the potential of peptide-based delivery for CRISPR action was described by Foss *et al*. (2023), where the A5K peptide was derived by fusing modified transactivator of transcription (TAT) and hemagglutinin 2 (HA2) peptides. This strategy achieved a 68 % knock-out rate upon delivering Cas9 RNP to T-cells (Foss *et al*., 2023). Another strategy is the use of hybrid nanoparticles formulated from both lipids and polymers. For example, Li *et al*. (2022) developed a self-assembling lipid-encased polymer for delivery of both pDNA and mRNA with improved long-term storage and gene delivery due to increased stability and sustained release from the hybrid complex. Additionally, high-throughput techniques are being developed for the formation of more efficient hybrid nanoparticles. Santhanes *et al*. (2022) demonstrated the use of microfluidics to produce a lipid and polymer hybrid nanoparticle through mixing of PLGA with DC-cholesterol and pDNA with mPEG2000-DSPE under consistent flow rates for consistently sized particles. Future work should not only improve the efficiency of these vectors but also consider the potential of multi-purpose strategies in which the delivery vector shows additionally functionalities. This strategy was explored by Qu *et al.* (2022) using a hybrid system with zeolitic imidazolate frameworks nanoparticles (ZIF-8), polydopamine, and a cationic antimicrobial peptide (LL37) to facilitate pDNA transfection while also providing antibacterial effects.

Cas endonuclease optimization

Further research on Cas endonucleases must be considered for improving the efficacy of CRISPR gene therapies in humans. For example, the advancement of CRISPR delivery methods to enable the use of multidomain type I CRISPR systems would allow distinct advantages such as increased editing specificity or the direct insertion of dsDNA (Zheng *et al*., 2020). Alternatively, protein engineering has been used to modify common Cas proteins to reduce their size, modify their electrostatic charges, augment their specificity, and improve their delivery and performance (Ferdosi *et al*., 2019; Schmidt *et al*., 2021; Xu *et al*., 2021). A clear example is the fusion of Cas endonuclease with base pair editors and reverse transcriptase to eliminate the need of a donor template in knock-in strategies, therefore increasing their clinical potential (Anzalone *et al*., 2019; Gaudelli *et al*., 2017).

Computational modeling of gene networks

CRISPR enables the targeting of genes linked to cell lineage commitment for directing stem and progenitor cell differentiation. In such processes, gene expression is interconnected in complex networks which tightly regulate each other to maintain appropriate levels of proteins and biological factors (Lin *et al*., 2022). Future CRISPR applications should consider these gene networks to best manipulate cell genetic profiles and biomanufacture more efficient cell therapies. To accomplish this, gene regulatory network (GRN) modeling, supported by advances in machine learning and artificial intelligence, offers detailed understanding of gene relationships and their impact on cell behavior (Pratapa *et al*., 2020). The understanding of these networks will allow the prediction of cell behavior following CRISPR gene editing and the determination of the most impactful gene targets. For example, Velazquez *et al*. (2021) used GRN modeling to identify the key transcription factors in liver tissues then subsequently applied CRISPRa to reprogram iPSCs into liver organoids. Detailed GRN models may also be applied to the implementation of synthetic gene circuits for the sustained or self-regulated manipulation of gene expression. Brunger *et al*. (2017a) achieved an autonomous negative feedback loop for preventing IL-1 and TNF-α induced inflammation in murine iPSCs, however this system repressed inflammatory cytokine levels below critical concentrations necessary for proper musculoskeletal regeneration. GRNs would allow for the design of more complex feedback systems to control expression levels more finely and appropriately.

Conclusion

CRISPR is a precise and tunable bio-inspired tool that allows for the understanding and

engineering of mammalian cell function. CRISPR systems can be used for the rapid knock-in, knock-out, activation, or inactivation of genes in progenitor cells or tissues to drive cell differentiation, treat genetic and degenerative diseases, or enhance tissue functions. CRISPR has advanced the engineering of bone, cartilage, and muscle and shows much promise for translation as well as novel therapies for the treatment of damaged tendons and ligaments. Despite this potential, CRISPR strategies are still limited by off-target editing, delivery difficulties, and low editing efficiencies. Future work must therefore focus on developing novel delivery approaches to offer more localized and safer gene editing, and to efficiently predict the effects of CRISPR modification for biomanufacturing more reliable and homogenous stem cell therapies with improved therapeutic effects.

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Author contributions

JPG, LCW and TGF planned the review and its outline. JPG, LCW and IF did the primary literature research and drafted the manuscript. JPG and TGF produced the figures. TGF edited the manuscript and provided funding. The manuscript was critically revised and approved by all authors.

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Conflict of interest

The authors declare no conflict of interest.

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