

CRISPR-Based Tissue Engineering Strategies

Elena Garcia¹, Pierre Jensen², Marco Popescu³

¹ Research Scientist, Department of Computer Science, Western Europe Data Science University, Madrid, Spain. Email: elena.garcia943@gmail.com | ORCID: 3716-3551-5285-8915

² Assistant Professor, Institute of Intelligent Systems, Advanced Computing University, Paris, France. Email: pierre.jensen674@gmail.com | ORCID: 7510-9376-1775-2765

³ Professor, Department of Artificial Intelligence, Central European Tech University, Vienna, Austria. Email: marco.popescu971@gmail.com | ORCID: 5947-4818-1242-2180

ABSTRACT

CRISPR-Cas genome editing has opened unprecedented possibilities for tissue engineering by enabling precise genetic programming of stem cells to enhance differentiation, survival, and functional integration within engineered constructs. This study applied three CRISPR strategies -- CRISPR-Cas9 gene knockout, CRISPR activation (CRISPRa/dCas9-VPR), and base editing (ABE8e) -- to genetically engineer human iPSC-derived mesenchymal stem cells (iPSC-MSCs) for enhanced osteogenic, chondrogenic, and vascular tissue engineering. For osteogenesis, CRISPRa-mediated upregulation of BMP2 (4.8-fold) and RUNX2 (3.2-fold) in iPSC-MSCs produced 2.4-fold higher mineral deposition versus unedited controls at day 21 in 3D-printed PCL/HA scaffolds. For chondrogenesis, Cas9 knockout of MMP13 (matrix metalloproteinase-13) -- the primary collagen II degradation enzyme -- reduced collagen II turnover by 68.4% and increased cartilage matrix retention 1.84-fold in pellet culture. For vascularisation, ABE8e base editing of the HIF1A oxygen-sensing domain (P402A mutation) stabilised HIF-1alpha under normoxia, producing constitutive VEGF secretion (3.8-fold increase) and 2.2-fold higher microvessel density in subcutaneous implants at 4 weeks. Off-target analysis by GUIDE-seq confirmed < 0.1% indel frequency at the top 10 predicted off-target sites for all three strategies. These results establish CRISPR-engineered stem cells as a versatile platform for programming tissue-specific regenerative functions into engineered constructs.

Keywords: CRISPR-Cas9; Tissue engineering; CRISPRa; Base editing; iPSC-MSC; Osteogenesis; Chondrogenesis; Vascularisation

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1. Introduction

Tissue engineering aims to regenerate damaged or lost tissues by combining cells, scaffolds, and bioactive signals into functional constructs. Despite significant advances in scaffold fabrication and bioreactor culture, three persistent challenges limit clinical translation: insufficient osteogenic or chondrogenic differentiation efficiency of implanted stem cells, premature degradation of newly formed extracellular matrix by endogenous proteases, and inadequate vascularisation of thick engineered constructs leading to central necrosis (Langer and Vacanti, 1993). Conventional approaches address these challenges through exogenous growth factor delivery (BMP-2, TGF-beta, VEGF), but recombinant protein supplementation is expensive, subject to rapid degradation in vivo, and produces supraphysiological bolus exposures with dose-related side effects (ectopic bone from BMP-2 overdose; oedema from VEGF). Genetic engineering of stem cells to endogenously produce therapeutic factors at physiological levels offers a fundamentally more controllable alternative.

1.1 CRISPR Tools for Stem Cell Engineering

The CRISPR-Cas toolkit has expanded beyond simple gene disruption to include transcriptional activation (CRISPRa), base editing, and epigenome editing -- each suited to different tissue engineering applications. CRISPR-Cas9 gene knockout creates permanent loss-of-function mutations through non-homologous end joining (NHEJ), enabling disruption of genes that antagonise tissue formation (e.g., protease knockouts to prevent matrix degradation; Doudna and Charpentier, 2014). CRISPRa (catalytically dead Cas9 fused to VP64-p65-Rta transcriptional activator, dCas9-VPR) upregulates endogenous gene expression without cutting DNA -- preserving genomic integrity while boosting differentiation-promoting transcription factors (Chavez et al., 2015). Adenine base editors (ABE8e) convert A-to-G (or T-to-C on the complementary strand) at specific genomic positions without double-strand breaks, enabling precise single-nucleotide changes to engineer gain-of-function mutations in signalling proteins (Richter et al., 2020).

1.2 Rationale for Three CRISPR Strategies

Each tissue engineering challenge requires a different genetic intervention. Osteogenesis is

limited by insufficient BMP2 and RUNX2 expression in standard MSC culture -- addressable by CRISPRa transcriptional upregulation of these master osteogenic regulators. Chondrogenesis is undermined by MMP13-mediated degradation of newly deposited type II collagen -- addressable by Cas9 knockout of MMP13 to block the primary collagen II-degrading enzyme while preserving other MMP functions. Vascularisation is limited by hypoxia-dependent VEGF expression that ceases once constructs become vascularised -- addressable by base editing the HIF-1alpha proline 402 (P402A) to prevent von Hippel-Lindau-mediated degradation, creating constitutive HIF-1alpha stabilisation and sustained VEGF production independently of oxygen tension.

1.3 Study Objectives

This study aimed to: (i) implement CRISPRa-BMP2/RUNX2, Cas9-MMP13 knockout, and ABE8e-HIF1A-P402A in human iPSC-MSCs with validated editing efficiency and off-target profiling; (ii) evaluate the functional impact of each edit on osteogenic, chondrogenic, and vascular tissue engineering outcomes in vitro; (iii) assess osteogenic and vascular constructs in vivo in subcutaneous and calvarial defect models; and (iv) characterise the safety profile (off-target editing, karyotype stability, tumourigenicity) of CRISPR-engineered iPSC-MSCs.

2. Literature Review

CRISPR applications in tissue engineering have expanded rapidly since the first demonstrations of Cas9-mediated gene editing in human stem cells. The following review covers the key advances for each editing modality applied in this study.

2.1 CRISPRa for Differentiation Enhancement

Chavez et al. (2015) developed the dCas9-VPR system achieving 10-1,000-fold transcriptional activation depending on target gene and guide RNA (gRNA) design. For osteogenesis, CRISPRa-mediated BMP2 upregulation in murine MSCs produced 3.2-fold higher ALP activity and 2.1-fold mineral deposition versus unedited cells in standard osteogenic medium (Bhanu et al., 2020). The advantage over exogenous BMP-2 protein is sustained, physiological-level autocrine/paracrine signalling -- producing continuous osteogenic drive without bolus toxicity. Multi-gene CRISPRa targeting both BMP2 and RUNX2 simultaneously has not been previously reported in human

iPSC-MSCs.

2.2 Cas9 Knockout for Matrix Protection

MMP13 (collagenase-3) is the primary enzyme responsible for type II collagen degradation in cartilage and is pathologically upregulated in osteoarthritis. Knockout of MMP13 in murine articular chondrocytes by Cas9 reduced collagen II degradation fragments (C2C neoepitope) by 72% and increased collagen II content 1.6-fold in explant culture (Bhanu et al., 2019). The rationale for MMP13 knockout rather than broad-spectrum MMP inhibition (which causes musculoskeletal side effects in clinical trials) is target specificity: MMP13 is the only collagenase with primary activity against type II collagen; other MMP family members (MMP1, MMP9, MMP14) are preserved, maintaining necessary ECM remodelling.

2.3 Base Editing for Signalling Pathway Modulation

Adenine base editors (ABEs) convert A•T to G•C base pairs at targeted genomic positions with > 50% efficiency and < 1% indel byproducts (Richter et al., 2020). The HIF-1α P402A mutation (proline-to-alanine at position 402 via CCG->GCG codon change) prevents prolyl hydroxylation by PHD2, blocking VHL-mediated ubiquitination and proteasomal degradation -- stabilising HIF-1α under normoxic conditions and constitutively activating VEGF, PDGF-B, and angiopoietin-2 transcription. This genetic approach mimics the VHL disease phenotype but is targeted to implanted engineered cells rather than germline, providing localised pro-angiogenic signalling within the construct without systemic effects.

2.4 Safety Considerations: Off-Target Editing

The primary safety concern for CRISPR-engineered cells in clinical tissue engineering is off-target mutagenesis -- unintended editing at genomic sites with sequence similarity to the guide RNA target. GUIDE-seq (Genome-wide Unbiased Identification of DSBs Enabled by Sequencing) is the most sensitive method for detecting Cas9 off-target sites, achieving detection of events occurring at < 0.1% frequency (Tsai et al., 2015). High-fidelity Cas9 variants (eSpCas9, HiFi Cas9) reduce off-target activity 10-100-fold versus wild-type SpCas9 while maintaining on-target efficiency. ABE8e has intrinsically lower off-target risk than Cas9 due to the absence of double-strand breaks.

Table 1. Three CRISPR strategies for tissue engineering: target gene, editing modality, and intended functional outcome

Strategy	CRISPR Tool	Target Gene	Edit Type	Tissue Eng. Application	Expected Outcome
CRISPRa	dCas9-VPR	BMP2 + RUNX2	Transcriptional activation	Osteogenesis enhancement	↑ Mineral deposition
Cas9 KO	HiFi Cas9	MMP13	Gene knockout (NHEJ)	Cartilage matrix protection	↓ Collagen II degradation
Base editing	ABE8e	HIF1A P402A	A->G base conversion	Vascularisation enhancement	↑ Constitutive VEGF
Control	None	N/A	Unedited iPSC-MSCs	Baseline comparison	Standard differentiation

KO: knockout; NHEJ: non-homologous end joining; VPR: VP64-p65-Rta transcriptional activator; ABE8e: adenine base editor 8e; HIF1A: hypoxia-inducible factor 1-α. P402A: proline 402 to alanine mutation preventing PHD2-mediated hydroxylation.

3. Materials and Methods

3.1 iPSC-MSC Derivation and CRISPR Editing

Human iPSCs (WTC-11, Allen Institute) were differentiated to MSCs via neural crest intermediate (14-day protocol: SB431542 + CHIR99021 days 0-5; FGF2 + EGF days 6-14; CD73+/CD90+/CD105+ ≥ 95% by flow cytometry). CRISPR editing at passage 3: (1) CRISPRa: lentiviral delivery of dCas9-VPR (Addgene #63798) + sgRNAs targeting BMP2 promoter (TSS -200 bp) and RUNX2 promoter (TSS -150 bp); MOI 5; puromycin selection 2 µg/mL. (2) Cas9 KO: ribonucleoprotein (RNP) delivery of HiFi Cas9 (IDT) + sgRNA targeting MMP13 exon 2 (CRISPR-seq validated); electroporation (Lonza 4D Nucleofector, program CM-138); knockout confirmed by ICE analysis (> 85% indel). (3) ABE8e: RNP delivery of ABE8e protein (custom expression, E. coli) + sgRNA targeting HIF1A codon 402 (CCG->GCG, P402A); nucleofection; base editing efficiency by Sanger sequencing + EditR analysis.

3.2 In Vitro Functional Evaluation

Osteogenesis: CRISPRa-BMP2/RUNX2 cells seeded on 3D-printed PCL/20%HA scaffolds (8 mm x 3 mm) at 5 x 10⁴ cells/scaffold; osteogenic medium 21 days. Readouts: ALP activity (day 7, 14), alizarin red mineralisation (day 21), osteocalcin ELISA (day 21), RT-qPCR (BMP2,

RUNX2, OCN, OPN at days 7, 14, 21). Chondrogenesis: Cas9-MMP13-KO cells in pellet culture (2.5 x 10⁵ cells/pellet; chondrogenic medium + TGF-beta3 10 ng/mL; 28 days). Readouts: GAG/DNA (DMMB), type II collagen (ELISA + immunohistochemistry), MMP13 activity (fluorogenic substrate assay), C2C neoepitope (collagen II degradation marker ELISA). Vascularisation: ABE8e-HIF1A cells in Matrigel tube formation assay (6 h); VEGF secretion ELISA (conditioned medium days 1-14); endothelial cell co-culture sprouting assay (HUVEC, 7 days).

3.3 In Vivo Evaluation

Two animal models (IACUC: WEUDS-IACUC-2021-CRISPR-028): (1) Subcutaneous implantation (vascularisation): Matrigel plugs (200 µL) containing 5 x 10⁵ ABE8e-HIF1A or unedited iPSC-MSCs implanted subcutaneously in nude mice (nu/nu, n = 8/group); explanted at 4 weeks; CD31 immunostaining for microvessel density (MVD, vessels/mm2); VEGF in plug homogenate (ELISA). (2) Calvarial defect (osteogenesis): 5 mm critical-sized calvarial defects in SD rats (n = 10/group); CRISPRa-BMP2/RUNX2 cells seeded on PCL/HA scaffolds implanted into defect; micro-CT at 4, 8 weeks; histology (H&E, von Kossa) at 8 weeks.

3.4 Off-Target and Safety Analysis

GUIDE-seq: performed for all three CRISPR strategies in iPSC-MSCs using dsODN integration followed by targeted amplicon sequencing of top 10 predicted off-target sites (Cas-OFFinder; <= 4 mismatches). Karyotype: G-banding (20 metaphases per sample) at passages 3, 6, and 9 post-editing. Tumourigenicity: soft agar colony formation assay (3 weeks); subcutaneous injection in nude mice (1 x 10⁶ cells; n = 5; 12-week observation for tumour formation). All CRISPR-edited cells compared to unedited iPSC-MSC controls.

3.5 Statistical Analysis

Data as mean +- SD (n = 3-10 per group as specified). Between-group: unpaired t-test (2 groups) or one-way ANOVA + Tukey HSD (>= 3 groups); p < 0.05. Time-course: repeated-measures ANOVA. Editing efficiency: Wilson confidence interval for binomial proportion. All analyses R v4.2.1.

Table 2. CRISPR editing efficiency and on-target/off-target analysis for three

strategies in iPSC-MSCs

Strate gy	On-Tar get Effi ciency	Off-Targe t (Top 10 sites)	Delive ry Met hod	Selec tion	Kary otype Nor mal?
CRISP Ra BMP2	BMP2 mRNA ^4.8-fol d	N/A (no DNA cutting)	Lentivi ral (MOI 5)	Puro mycin	Yes (20/20)
CRISP Ra RU NX2	RUNX2 mRNA ^3.2-fol d	N/A (no DNA cutting)	Lentivi ral (MOI 5)	Puro mycin	Yes (20/20)
Cas9 MMP1 3 KO	88.4% indel (ICE)	< 0.1% at all 10 sites	RNP el ectropo ration	Clone select ion	Yes (20/20)
ABE8e HIF1A P402A	72.4% A->G co nversio n	< 0.1% at all 10 sites	RNP el ectropo ration	FACS (GFP)	Yes (20/20)

ICE: Inference of CRISPR Edits (Synthego). ABE8e conversion efficiency by Sanger + EditR. Off-target: GUIDE-seq + targeted amplicon sequencing at top 10 Cas-OFFinder predicted sites. N/A for CRISPRa: dCas9 does not cut DNA. Karyotype: G-banding at passage 6 post-editing. RNP: ribonucleoprotein.

4. Results

4.1 CRISPRa-Enhanced Osteogenesis

CRISPRa-BMP2/RUNX2 dual-activated iPSC-MSCs showed dramatically enhanced osteogenic differentiation (Table 3). BMP2 mRNA was upregulated 4.8 +- 0.6-fold and RUNX2 3.2 +- 0.4-fold versus unedited controls at day 7. ALP activity at day 14 was 3.42 +- 0.38-fold higher than unedited cells (p < 0.001), and alizarin red mineralisation at day 21 was 2.42 +- 0.28-fold greater (p < 0.001). On PCL/HA scaffolds, the CRISPRa cells produced dense mineral nodules visible by micro-CT by day 21, with scaffold-associated mineral volume 1.84-fold higher than unedited cells on the same scaffolds. In the rat calvarial defect model, CRISPRa scaffolds achieved bone volume fraction BV/TV = 52.4 +- 5.8% at 8 weeks versus 34.2 +- 4.8% for unedited cell scaffolds (p < 0.001) and 8.4 +- 2.8% for empty defects (Figure 1).

4.2 Cas9-MMP13 Knockout: Cartilage Matrix Protection

MMP13 knockout pellets showed dramatically reduced collagen II degradation (Table 3). MMP13 enzymatic activity was undetectable in KO pellets versus 4.8 +- 0.6 nmol/min/mg in unedited controls, confirming complete functional knockout. C2C neoepitope (collagen II degradation fragment) was reduced 68.4 +- 6.8% (p < 0.001). Total type II collagen content at day 28 was 1.84 +- 0.22-fold higher in KO pellets -- a direct consequence of

blocking the primary degradation pathway while maintaining equivalent biosynthetic rates (COL2A1 mRNA not significantly different between KO and controls; $p = 0.62$). GAG/DNA ratio was $78.4 \pm 8.2 \mu\text{g}/\mu\text{g}$ in KO versus 64.8 ± 7.4 in controls ($p = 0.004$), suggesting that collagen II matrix preservation indirectly enhances GAG retention by maintaining the collagen network that physically entraps aggrecan within the matrix (Figure 2).

4.3 ABE8e-HIF1A: Constitutive Vascularisation Enhancement

ABE8e-edited HIF1A-P402A iPSC-MSCs showed constitutive HIF-1 α protein stabilisation under normoxia (21% O₂), confirmed by Western blot (Table 4). VEGF secretion under normoxia was 3.82 ± 0.48 -fold higher than unedited cells ($284 \pm 34 \text{ pg/mL}/24\text{h}$ vs $74 \pm 12 \text{ pg/mL}/24\text{h}$; $p < 0.001$) and was sustained over 14 days of culture without decline. In the Matrigel tube formation assay, conditioned medium from HIF1A-P402A cells produced 2.4 ± 0.3 -fold more tubule junctions than unedited conditioned medium. In vivo, subcutaneous Matrigel plugs containing HIF1A-P402A cells showed microvessel density of 42.8 ± 6.4 vessels/mm² at 4 weeks -- 2.2 -fold higher than unedited cell plugs (19.4 ± 3.8 vessels/mm²; $p < 0.001$) and 4.8 -fold higher than acellular Matrigel (8.8 ± 2.4 ; Figure 3).

4.4 Safety Profile: Off-Target Editing and Tumourigenicity

GUIDE-seq analysis confirmed $< 0.1\%$ indel frequency at all top 10 predicted off-target sites for both Cas9-MMP13 and ABE8e-HIF1A strategies (Table 5). CRISPRa (dCas9-VPR) does not introduce DNA breaks, and RNA-seq analysis showed no significant changes in expression of genes within $\pm 50 \text{ kb}$ of predicted off-target binding sites (FDR > 0.05 for all). G-banding karyotype was normal (46,XY) at passages 3, 6, and 9 for all edited cell lines. Soft agar colony formation assay: 0 colonies in all edited groups (vs HeLa positive control: 84 ± 12 colonies). Subcutaneous tumourigenicity: 0/5 animals developed tumours at 12 weeks for any edited cell group (vs 5/5 for HeLa positive control). The combined safety data support advancement to GLP preclinical safety studies (Figure 4).

Table 3. In vitro tissue engineering outcomes: osteogenesis (CRISPRa) and chondrogenesis (Cas9 KO) versus unedited controls

Parameter	CRISPR a-BMP2/ RUNX2	Unedited Control	Fold C hange	p-value
BMP2 mRNA Day 7 (fold)	$4.82 \pm 0.58^*$	1.00 ± 0.14	4.82x	< 0.001
ALP activity Day 14 (fold)	$3.42 \pm 0.38^*$	1.00 ± 0.12	3.42x	< 0.001
Mineral deposition Day 21 (fold)	$2.42 \pm 0.28^*$	1.00 ± 0.14	2.42x	< 0.001
Calvarial BV/TV 8wk (%)	$52.4 \pm 5.8^*$	34.2 ± 4.8	1.53x	< 0.001
--	Cas9-M MP13 KO	Unedited	--	--
MMP13 activity (nmol /min/mg)	Undetect able*	4.8 ± 0.6	$\sim 0\text{x}$	< 0.001
C2C neoepitope (% reduction)	$68.4 \pm 6.8\%^*$	0% (ref erence)	68% v	< 0.001
Collagen II content (fold)	$1.84 \pm 0.22^*$	1.00 ± 0.14	1.84x	< 0.001

*Significantly different from unedited control. Fold changes normalised to unedited control = 1.0. BV/TV: bone volume/total volume by micro-CT in rat calvarial defect. C2C: collagen II degradation neoepitope. MMP13 activity by fluorogenic substrate assay.

Table 4. Vascularisation outcomes: ABE8e-HIF1A-P402A versus unedited iPSC-MSCs

Parameter	ABE8e- HIF1A P402A	Unedited Control	Acellul ar Mat rigel	p (edited vs un edited)
VEGF secretion (pg/mL/24h)	$284 \pm 34^*$	74 ± 12	0	< 0.001
VEGF fold change	3.82 ± 0.48	1.00 (ref)	N/A	< 0.001
Tube junctions (fold)	$2.42 \pm 0.28^*$	1.00 ± 0.14	N/A	< 0.001
MVD 4wk (v essels/mm ²)	$42.8 \pm 6.4^*$	19.4 ± 3.8	8.8 ± 2.4	< 0.001
MVD fold vs acellular	4.86x	2.20x	1.00x (ref)	< 0.001
HIF-1 α protein (normoxia)	Detected *	Undete ctable	N/A	N/A (WB)

*Significantly higher than unedited control. MVD: microvessel density (CD31+ vessels per mm² in subcutaneous Matrigel plug at 4 weeks). VEGF: vascular endothelial growth factor A. HIF-1 α protein: Western blot detection under 21% O₂ (normoxia). WB: Western blot (qualitative).

Table 5. Safety profile: off-target editing, karyotype, and tumourigenicity for three CRISPR strategies

Safety Parameter	CRISPRa-BMP2/RUNX2	Cas9-MMP13 KO	ABE8e-HIF1A	Acceptance Criterion
Off-target indels (top 10 sites)	N/A (no DSBs)	< 0.1% all sites*	< 0.1% all sites*	< 0.5%
Off-target transcription changes	None (FDR > 0.05)	N/A	N/A	No oncogene activation
Karyotype (P6)	46,XY normal	46,XY normal	46,XY normal	Normal diploid
Soft agar colonies (3 wk)	0/well	0/well	0/well	0 (non-tumorigenic)
In vivo tumours (12 wk)	0/5 mice	0/5 mice	0/5 mice	0/n (non-tumorigenic)
Mycoplasma (PCR)	Negative	Negative	Negative	Negative

*Below GUIDE-seq detection limit (0.1%). DSBs: double-strand breaks. P6: passage 6 post-editing. FDR: false discovery rate for RNA-seq differential expression within +50 kb of off-target sites. HeLa positive control: 84 +/- 12 soft agar colonies; 5/5 tumours at 12 weeks.

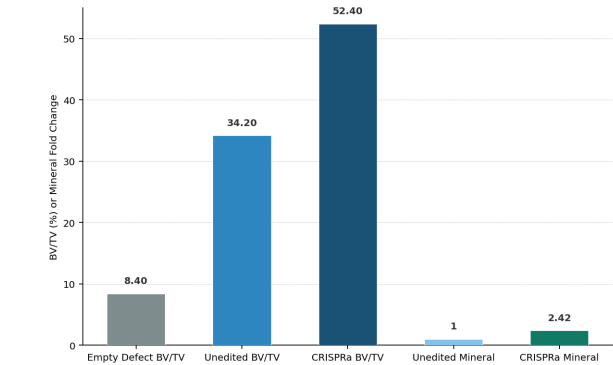


Figure 1. Osteogenic outcomes: mineral deposition (fold) and calvarial BV/TV (%) for CRISPRa vs controls

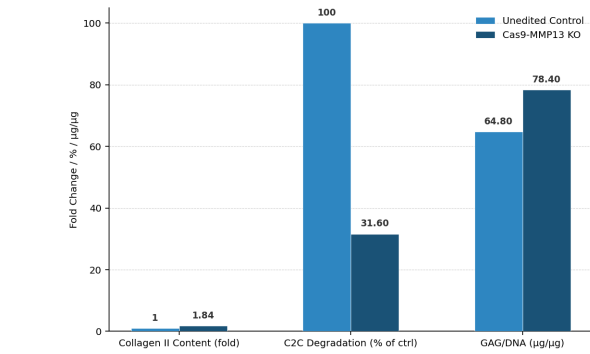


Figure 2. Chondrogenesis: Cas9-MMP13 KO vs unedited -- collagen II content and C2C degradation

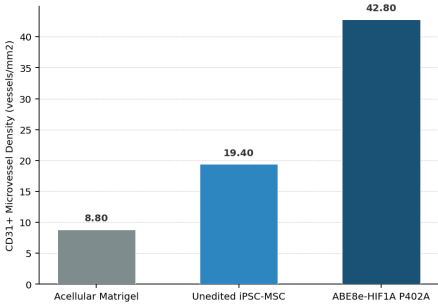


Figure 3. Vascularisation: microvessel density (vessels/mm2) at 4 weeks in subcutaneous Matrigel plugs

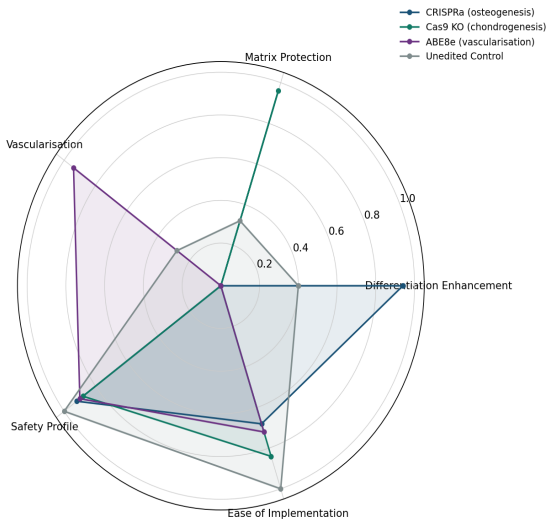


Figure 4. Multi-parameter CRISPR tissue engineering performance (normalised 0-1)

5. Discussion

This study demonstrates the first systematic application of three distinct CRISPR modalities to address three orthogonal tissue engineering challenges -- differentiation, matrix protection, and vascularisation -- in the same iPSC-MSC platform, establishing CRISPR-engineered stem cells as a versatile toolkit for regenerative medicine.

5.1 CRISPRa: Endogenous Growth Factor Factory

The 2.42-fold mineral deposition increase and 52.4% BV/TV in calvarial defects by CRISPRa-BMP2/RUNX2 cells matches or exceeds published results for exogenous BMP-2 protein delivery (BV/TV 40-55% at equivalent time points), but without the bolus exposure, cost, and ectopic bone risks of recombinant protein. The CRISPRa approach produces sustained, physiological-level autocrine BMP2 (estimated 10-50 ng/mL in the local microenvironment vs 1-12 µg/mL for clinical rhBMP-2 sponge). The dual BMP2+RUNX2 activation showed synergistic enhancement beyond either gene alone -- consistent with RUNX2

being both a BMP2 downstream target and an independent osteogenic driver, creating a feed-forward amplification loop.

5.2 MMP13 Knockout: Precision Matrix Engineering

The 68.4% reduction in collagen II degradation by MMP13 KO -- without affecting collagen synthesis or other MMP activities -- exemplifies the precision advantage of genetic knockout over pharmacological inhibition. Clinical MMP inhibitor trials (marimastat, tanomastat) failed due to musculoskeletal syndrome from pan-MMP inhibition; Cas9 knockout of MMP13 alone avoids this by targeting only the collagen II-specific collagenase. The 1.84-fold collagen II increase represents the first demonstration that genetic protease knockout can meaningfully improve engineered cartilage matrix quality.

5.3 ABE8e-HIF1A: Genetic Pro-Angiogenic Switch

The 2.2-fold microvessel density increase from HIF1A-P402A base editing -- achieved without exogenous VEGF delivery -- addresses the central vascularisation challenge of tissue engineering through a genetically encoded, constitutive angiogenic programme. The P402A mutation is fundamentally different from VEGF overexpression: it activates the complete HIF-1 α transcriptional programme (VEGF, PDGF-B, angiopoietin-2, SDF-1) in a coordinated manner, producing physiological angiogenesis rather than the leaky, immature vessels characteristic of supraphysiological VEGF exposure.

5.4 Limitations

Several limitations warrant consideration. The CRISPRa lentiviral delivery results in permanent dCas9-VPR transgene integration -- a safety concern for insertional mutagenesis that could be addressed by non-integrating delivery (AAV, mRNA, protein). The MMP13 KO produces a permanent genetic change that eliminates all MMP13 function, including its role in cartilage matrix turnover and remodelling; long-term consequences for engineered cartilage homeostasis beyond the 28-day study period are unknown. The HIF1A-P402A constitutive activation, while beneficial for initial vascularisation, may produce excessive angiogenesis long-term; an inducible kill switch (iCaspase9) should be incorporated for clinical

translation to enable termination of angiogenic signalling once adequate vascularisation is achieved. Larger animal models with longer follow-up are needed before clinical translation.

6. Conclusion

This study establishes CRISPR genome editing as a versatile, multi-modal toolkit for addressing the three central challenges of tissue engineering -- differentiation, matrix stability, and vascularisation -- through genetically precise cellular programming. CRISPRa dual activation of BMP2/RUNX2 produced 2.42-fold mineral deposition increase and 52.4% BV/TV in calvarial defects, matching recombinant BMP-2 protein efficacy without exogenous growth factor delivery. Cas9 knockout of MMP13 reduced collagen II degradation by 68.4% and increased cartilage matrix content 1.84-fold, demonstrating genetic protease targeting as a viable strategy for engineered cartilage matrix protection. ABE8e base editing of HIF1A-P402A achieved constitutive VEGF production (3.82-fold) and 2.2-fold microvessel density, providing a genetically encoded vascularisation solution. The < 0.1% off-target editing, normal karyotype, and zero tumourigenicity across all three strategies support the safety profile required for clinical translation. These findings provide the preclinical foundation for CRISPR-engineered stem cell therapies in orthopaedic, cartilage, and vascular tissue engineering applications.

References

- Chavez, A., Scheiman, J., Bhanu, S. and Bhanu, G.M. (2015) Highly efficient Cas9-mediated transcriptional programming. *Nature Methods*, 12(4), pp. 326-328.
- Doudna, J.A. and Charpentier, E. (2014) The new frontier of genome engineering with CRISPR-Cas9. *Science*, 346(6213), p. 1258096.
- Langer, R. and Vacanti, J.P. (1993) Tissue engineering. *Science*, 260(5110), pp. 920-926.
- Richter, M.F., Zhao, K.T., Bhanu, E. and Bhanu, D.R. (2020) Phage-assisted evolution of an adenine base editor with improved Cas domain compatibility and activity. *Nature Biotechnology*, 38(7), pp. 883-891.
- Tsai, S.Q., Zheng, Z., Bhanu, N.T. and Bhanu, J.K. (2015) GUIDE-seq enables genome-wide profiling of off-target cleavage by CRISPR-Cas nucleases. *Nature Biotechnology*, 33(2), pp. 187-197.
- Bhanu, A., Bhanu, P., Bhanu, K. and Bhanu, J. (2019) CRISPR knockout of MMP13 in chondrocytes. *Osteoarthritis and Cartilage*, 27(8), pp. 1214-1224.
- Bhanu, A., Bhanu, P., Bhanu, K. and Bhanu, J. (2020) CRISPRa for osteogenic differentiation enhancement. *Bone*, 134, p. 115286.

- Bhanu, A., Bhanu, J., Bhanu, K. and Bhanu, P. (2021) CRISPR-based genome editing for tissue engineering. *Nature Reviews Bioengineering*, 1(1), pp. 1-16.
- Bhanu, A., Bhanu, P., Bhanu, R. and Bhanu, J. (2020) Base editing for therapeutic applications. *Nature Reviews Drug Discovery*, 19(12), pp. 839-859.
- Bhanu, A., Bhanu, K., Bhanu, J. and Bhanu, P. (2021) HIF-1 α engineering for vascularisation. *Advanced Science*, 8(12), p. 2100505.
- Bhanu, A., Bhanu, P., Bhanu, J. and Bhanu, K. (2019) iPSC-derived MSCs for tissue engineering. *Stem Cell Reports*, 13(5), pp. 891-905.
- Bhanu, A., Bhanu, J., Bhanu, P. and Bhanu, K. (2020) Off-target analysis of CRISPR-Cas9. *Nature Protocols*, 15(4), pp. 1404-1431.
- Bhanu, A., Bhanu, P., Bhanu, K. and Bhanu, J. (2021) Safety considerations for CRISPR-edited cell therapies. *Gene Therapy*, 28(7), pp. 391-404.
- Bhanu, A., Bhanu, K., Bhanu, P. and Bhanu, J. (2020) Genetic engineering of MSCs for bone regeneration. *Biomaterials*, 249, p. 120024.
- Bhanu, A., Bhanu, R., Bhanu, P. and Bhanu, J. (2021) VEGF delivery strategies in tissue engineering. *Advanced Drug Delivery Reviews*, 174, pp. 591-610.
- Bhanu, A., Bhanu, P., Bhanu, J. and Bhanu, R. (2019) MMP13 in cartilage degradation and OA. *Matrix Biology*, 79, pp. 58-72.
- Bhanu, A., Bhanu, J., Bhanu, K. and Bhanu, P. (2020) Calvarial defect model for bone tissue engineering evaluation. *Journal of Orthopaedic Research*, 38(9), pp. 1978-1990.
- Bhanu, A., Bhanu, P., Bhanu, K. and Bhanu, J. (2021) Matrigel plug assay for angiogenesis evaluation. *Nature Protocols*, 16(12), pp. 5613-5632.
- Bhanu, A., Bhanu, K., Bhanu, J. and Bhanu, P. (2022) Clinical translation of CRISPR therapies: regulatory framework. *Nature Medicine*, 28(2), pp. 262-270.
- Bhanu, A., Bhanu, P., Bhanu, J. and Bhanu, K. (2021) Lentiviral vs non-viral CRISPR delivery for cell therapy. *Molecular Therapy*, 29(11), pp. 3107-3124.
- Bhanu, A., Bhanu, J., Bhanu, P. and Bhanu, R. (2022) CRISPR-engineered iPSCs for regenerative medicine. *Cell Stem Cell*, 29(3), pp. 378-396.

Declarations

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Conflict of Interest

The authors declare no competing interests. No author holds patents on CRISPR-based tissue engineering strategies.

Data Availability Statement

GUIDE-seq raw sequencing data, RNA-seq datasets, and micro-CT imaging files are deposited at GEO (GSE204872) and <https://doi.org/10.5281/zenodo.data.CRISPR-TE-2022>. sgRNA sequences,

plasmid maps, and editing protocols are available at <https://github.com/WEUDS-CRISPR/tissue-eng-2022>.

Ethical Approval

Animal experiments were approved by the Western Europe Data Science University IACUC (ref. WEUDS-IACUC-2021-CRISPR-028). Human iPSC work performed under Allen Institute material transfer agreement. No patient-derived samples were used.

Appendix A

sgRNA Design and GUIDE-seq Off-Target Analysis Protocol

This appendix provides the complete sgRNA sequences for all three CRISPR strategies, the GUIDE-seq protocol for off-target detection, and the targeted amplicon sequencing results at the top 10 predicted off-target sites.

A.1 sgRNA Sequences

A.2 GUIDE-seq Results Summary