Osteoarthritis and Cartilage 27 (2019) 140–147

Osteoarthritis and Cartilage

CRISPR-Cas9 targeting of MMP13 in human chondrocytes leads to significantly reduced levels of the metalloproteinase and enhanced type II collagen accumulation

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ARTICLE INFO

Article history:
Received 30 April 2018
Accepted 4 September 2018

Keywords:
CRISPR-Cas9
Autologous chondrocyte implantation
MMP13
Cartilage
3D spheroids

SUMMARY

Objective: To investigate the efficacy of CRISPR-Cas9 mediated editing in human chondrocytes, and to develop a genome editing approach relevant to cell-based repair.
Methods: Transfection of human articular chondrocytes (both healthy and osteoarthritic) with ribonucleoprotein complexes (RNP) containing Cas9 and a crisprRNA targeting exon2 of MMP13 was performed to assess editing efficiency and effects on MMP13 protein levels and enzymatic activity. Using spheroid cultures, protein levels of a major target of MMP13, type II collagen, were assessed by western blot and immunofluorescence.
Results: With an editing efficiency of 63–74%, secreted MMP13 protein levels and activity were significantly reduced (percentage decrease 34.14% without and 67.97% with IL-1β based on median values of MMP13 enzymatic activity, N = 7) comparing non-edited with edited cell populations using an exon-targeting gRNA resulting in premature stop codons through non-homologous end joining (NHEJ). Accumulation of cartilage matrix protein type II collagen was enhanced in edited cells in spheroid culture, compared to non-edited controls.
Conclusion: CRISPR-Cas9 mediated genome editing can be used to efficiently and reproducibly establish populations of human chondrocytes with stably reduced expression of key genes of interest without the need for clonal selection. Such an editing approach has the potential to greatly enhance current cell-based therapies for cartilage repair.

Introduction

Genome editing – the introduction of targeted genomic sequence changes in isolated cells or whole organisms is an extremely powerful research tool with huge potential for clinical application and regenerative medicine[1–3]. Cas9 is a nuclease directed by guide RNAs (gRNAs) to complementary sites in the genome. It induces double strand breaks (DSBs) which repair through non-homologous end-joining (NHEJ), or in the presence of a suitable DNA template, by homology-directed repair (HDR). The CRISPR-Cas9 system thus enables highly specific gene editing giving it great potential for correction of genetic (particularly monogenic) diseases[4,5], or for revolutionising cell-based therapies. Genome editing of the germline in humans is obviously fraught with ethical considerations. *In situ* editing through delivery of Cas9 would also have major issues with regard to both efficacy and safety. However, genome editing of human cells *ex vivo* for subsequent re-implantation is a realistic avenue for clinical application.

Maintenance of the articular cartilage which lines and protects our joints is a balance between production and breakdown of the tissue. With age cartilage progressively thins, indicating a gradual imbalance in this process that predisposes to injury and joint diseases such as arthritis. Articular cartilage, lacking a blood and nerve supply has a relatively weak endogenous repair response[6,7]. An established cell therapy for cartilage repair, called autologous chondrocyte implantation (ACI), involves surgical removal of a small amount of unaffected cartilage from the injured joint, extraction and culture-expansion of the chondrocyte cells *in vitro*, before re-implantation at the injured site in a second surgical

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https://doi.org/10.1016/j.joca.2018.09.001
1063-4584/

procedure[8,9]. We proposed that the *in vitro* phase of this procedure would be amenable to a genome editing approach, where desired edits could be introduced before cell expansion and subsequent re-implantation. We tested the feasibility of such an editing strategy in the present study.

In the absence of a DNA repair template gRNA-specified sites of Cas9 cleavage are repaired through NHEJ, creating insertions or and deletions (indels) which – if in the open reading frame (ORF) – can lead to frameshift mutations and premature stop codons[10]. We adopted this approach to target MMP13 in primary human chondrocytes using ribonucleoprotein delivery of Cas9 and gRNA. MMP13 is the major collagenase found in cartilage and its upregulation has been implicated in osteoarthritis[11,12]. We demonstrate high editing efficiency in human chondrocytes, resulting in dramatically reduced levels of MMP13 protein and activity and enhanced levels of major matrix protein type II collagen. Our editing strategy (see Fig. 1) is feasible for clinical application as it does not involve the lengthy procedure of clonal selection and subsequent expansion.

Materials and methods

Human articular chondrocyte (HAC) isolation and culture

Normal healthy knee articular cartilage (age range 21–66 years; mean ± SD of 45 ± 13.9 years; six males, three females) was obtained from amputations due to sarcomas not involving the joint space as previously described[16]. Osteoarthritic cartilage samples (age range 52–89 years; mean ± SD of 69.7 ± 10.0 years; 16 males, four females) were obtained from patients undergoing total or unilateral knee replacement. Tissue samples were obtained from the Oxford Musculoskeletal Biobank and were collected with informed donor consent in full compliance with national and institutional ethical requirements, the United Kingdom Human Tissue Act, and the Declaration of Helsinki (HTA Licence 12,217 and Oxford REC C 09/H0606/11). Chondrocytes were isolated as described previously[17].

Transfection with ribonucleoprotein (RNP) complexes

HACs from both healthy and osteoarthritic (OA) affected individuals were transfected at either P0 or P1 in 6-well or 12-well format once they reached at least 50% confluence. *Streptococcus pyogenes* Cas9-3NLS protein (#1074181) and all crispr and tracr (#1072532) RNAs as well as negative control (#1072544) crispr RNAs were purchased from Integrated DNA Technologies. The sequence for crRNA in exon2 of MMP13 is: UAGGAUGGUAGUAUGAUCUC. Crispr and tracr RNA were resuspended in Tris–EDTA buffer, pH 7.5, and combined in equimolar ratios annealing buffer at a final concentration of 10 μM. For delivery of ribonucleoprotein complexes (RNP)s, Lipofectamine™ CRISPRMAX™ Cas9 Transfection Reagent (Thermo Fisher, CMAX00008) was used. Cells were fed with 1 ml (12 well format) or 2 ml (6 well format) 2–4 h before transfection with 1× Dulbecco's modified Eagle medium (DMEM)/10% fetal bovine serum (FBS). Prior to each transfection, the required amount of crispr/tracrRNA was denatured for 5 min at 95°C, followed by cooling to room temperature for another 5 min in order to allow for crispr/tracr annealing. For each well of a 12 well plate, 1 μg Cas9-3NLS was added to 50 μl Optimem in a 1.5 ml Eppendorf tube, followed by 400 ng of denatured/annealed crispr–tracr RNA, and 2 μl Cas9 PLUS reagent. Complexes were allowed to form for 15 min at room temperature. In a separate 1.5 ml Eppendorf tube, 3 μl Lipofectamine CRISPRMAX was added to 50 μl Optimem and incubated for 2 min. Complexed RNPs (Cas9 protein and crispr–tracr RNA) were added to the tube with diluted Lipofectamine CRISPRMAX and incubated for another 15 min, then complexes were added dropwise to wells. Transfections with non-targeting control contained the exact same amount of transfection components as the MMP13 wells except for having a crispr RNA

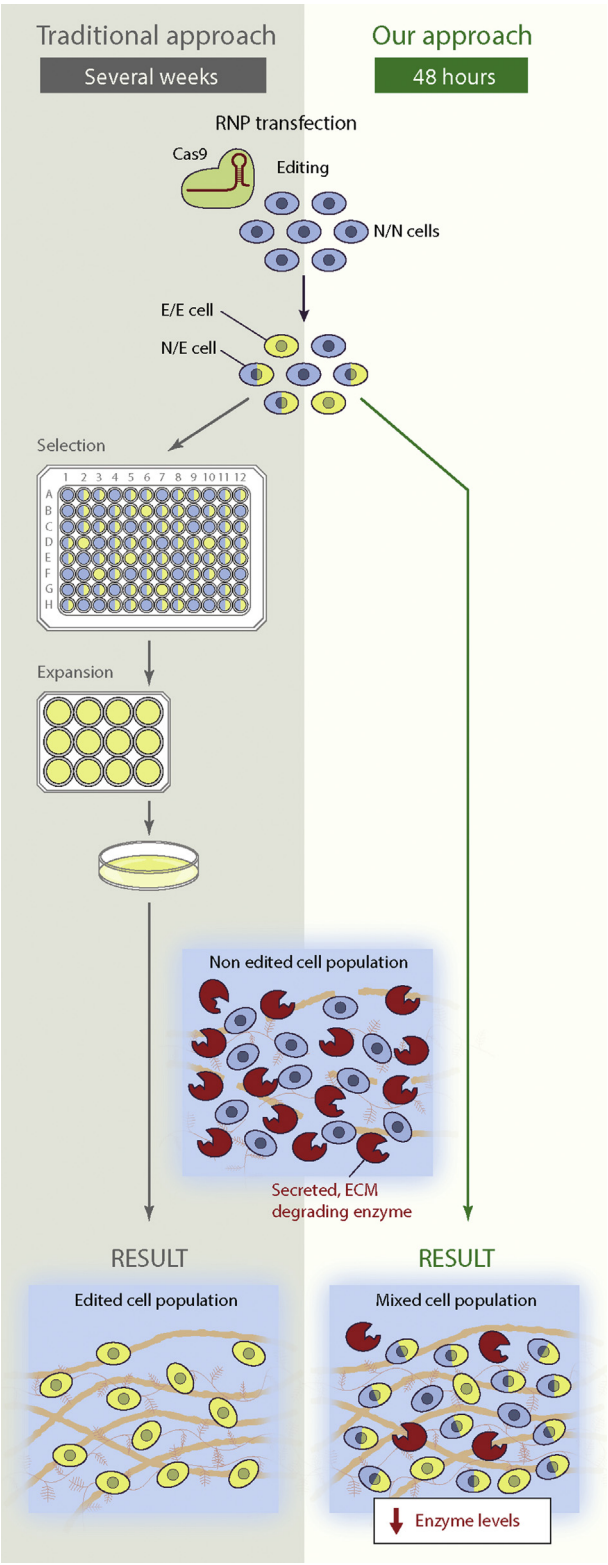

Fig. 1. Cas9-editing strategy for human chondrocytes. ribonucleoprotein complexes (RNP), Ribonucleoprotein; N, non-edited; E, edited; ECM, extracellular matrix; KO knock-out.

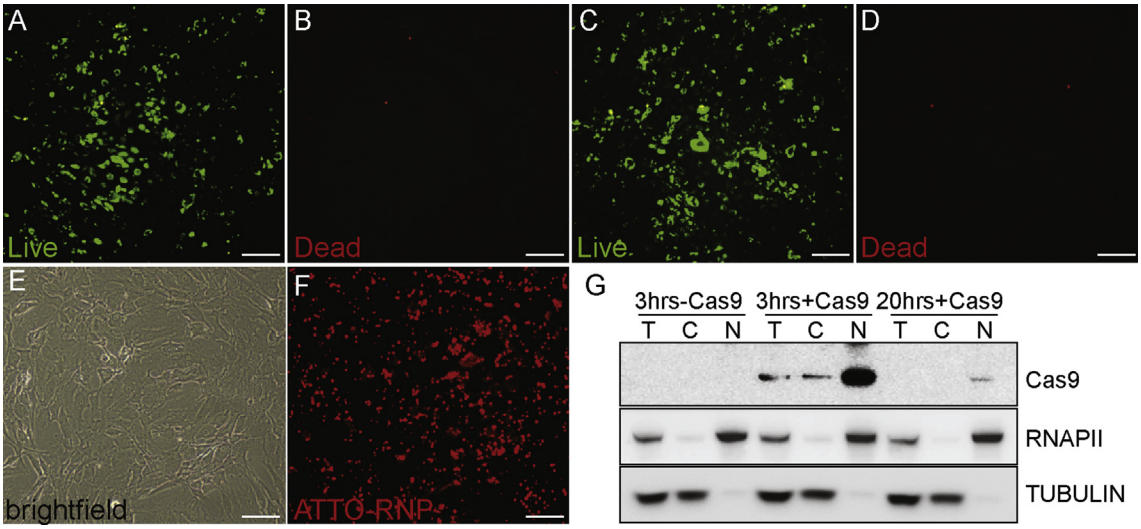

Fig. 2. RNP transfection of Human Articular Chondrocyte (HAC)s displays high efficiency and low toxicity. HACs at P1 in 12 well plates were left untreated (A, B) or transfected with MMP13 targeting Ribonucleoprotein complexes (RNPs, C, D) and a live-dead assay was performed 24 h post transfection. Transfection efficiency was monitored with fluorescently labelled tracrRNA within the RNP complex and uptake was visualized 6 h post transfection (E, F). To demonstrate Cas9 delivery and localization, cells transfected with RNPs containing Cas9 or without Cas9 were harvested 3 h and 20 h after transfection, separated into cytoplasmic and nuclear fractions and immunoblotted for Cas9, RNAPII and Tubulin (G). T, total cell fraction; N, nuclear fraction; C, cytoplasmic fraction. Scale bar = 100 μm.

with no known target sequence in the human genome. For Fig. 2(F), cells were transfected with RNPs formed with sgRNA containing ATTO-labelled tracr RNA (IDT #1075927).

Cytoplasmic/nuclear fractionation

Cytoplasmic and nuclear fractionation of HAC was carried out using the NE-PER kit (Thermo Fisher 78833) from confluent 6 well plates according to the manufacturer's instructions.

IL-1β induction

At a minimum of 90% confluence edited and non-edited cells were washed twice with warm Dulbecco's phosphate buffered saline (DPBS), and fresh media (1× DMEM, 1× insulin-transferrin-selenium (ITS), Thermo Fisher 41400-045) supplemented with 0.1 ng/ml IL-1β (Peprotech, 200-01B) was added. Supernatants and cells were harvested 20–22 h post IL-1β induction.

DNA/RNA/protein purification

Nucleic acids were purified using the ZR-Duet™ DNA/RNA MiniPrep kit (Zymo Research, #D7001). RNA from spheroids was prepared with TRIzol™ Reagent (Thermo Fisher, #15596018) according to the manufacturer's instructions. 300 μl Trizol was used per five spheroids and vortexed for around 10 min until spheroids had dissolved.

T7E1 assay

The region of interest surrounding the edited site of human MMP13 was amplified for 40 cycles using 15–25 ng genomic DNA and 2× Q5 Hot Start polymerase (New England Biolabs, Q5 Hot Start High-Fidelity 2× master mix, M0494S) in a 15 μl reaction volume with primer C61 (5-ATCAGGAACCCCGCATCTTG-3) and C62 (5-CGGAAAGACAACAGTCCCCA-3). 5 μl non-purified polymerase chain reaction (PCR) product in 1× NEB2 a total volume of 11 μl was denatured and reannealed in a thermocycler using following protocol: initial denaturation 95°C, 5 min; annealing 95°–85°C ramp rate (−2°C/s), 85°–25°C at −0.1°C/s. After reannealing, 2 units T7E1 (New England Biolabs, T7 Endonuclease I, M0302S) were added to the PCR product and incubated for 45–60 min at 37°C. Digestion products were separated over 2.5% agarose containing SYBR Safe gel stain (S33102, Thermo Fisher). Off target primers are as follows: CNOT6-FWD, CAGGTGCTAGCAACAGACAA; CNOT6-REV, GGGCTAACAAAACCCTAAGAGTG; OT2-FWD, AGTTCTTGCCTGGCTGTACT; OT2-REV, GAGAGTACAATGGTGGCTGC; OT3-FWD, CAATCCAGACTCCCGCCAATA; OT3-REV, TAGGTCTCGCCCTGATGGG.

Subcloning of PCR products

For sequencing of PCR products of MMP13-exon2 edited cells, purified PCR products were subcloned into Zero Blunt® PCR Cloning Kit (Thermo Fisher, K2700-20) and spread on LB-Kanamycin plates. For each donor, 24 colonies were picked and grown o/n in 3 ml LB-Kan. Plasmids were prepped using Zyppy™ Plasmid Miniprep Kit (Zymo Research, D4036) and sent for sequencing.

Western blotting

Supernatants collected from cells were precipitated using 6.1 N Trichloroacetic acid solution (250 μl per 1 ml supernatant (SN), Sigma T0699-100 ML). Pellets were washed with ice cold acetone, air-dried and dissolved in solubilisation buffer (SB, 50 mM Tris–HCl pH6.8, 100 mM DTT, 2% SDS). Spheroids were solubilized directly in SB.

All samples were denatured for 5 min at 95–100°C before loading onto 4–12% Bis-Tris (Thermo Fisher, NP0336BOX) or 3–8% Tris–Acetate (Thermo Fisher, #EA03752BOX) gels. Antibodies: MMP13 (abcam ab39012, 1:1000), COL2A1 (Chondrex #7005, 1:1000), MMP1 (abcam, ab134184), Tubulin (abcam ab7291, 1:10,000), Transferrin-HRP (abcam ab185070, 1:5000), Cas9 (Active Motif 61,578, 1:1000), RNAPII (Sigma, component of CHP1 kit).

RT and qPCR

300–800 ng RNA was treated with TURBO DNA-free™ Kit (Thermo Fisher AM1907). Reverse transcription was carried out using the High-Capacity cDNA Reverse Transcription Kit, (Thermo

Fisher, 4368814) with RNase Inhibitor (Thermo Fisher N8080119). SYBR qPCR was performed using the Fast SYBR™ Green Master Mix (Thermo Fisher, 4385612) on a Viia7 theromcycler and normalized to RPLP0. Following primers were used: RPLP0-FWD, CCATTGAAATCCTGAGTGATGTG; RPLP0-REV, CTTCGCTGGCTCCCACTTT; MMP13-FWD, AGACTTCCCAGGAATTGGTGA; MMP13-REV, ACGGTTACTCCAGATGCTGT; MMP1-FWD, CCAGGTATTGGAGGGGATGC; MMP1-REV, GTCCAAGAGAATGGCCGAGT; MMP8-FWD, CCATGTGCAGATTTCCAAGGC; MMP8-REV, AGACTGATACTGGTTGCTTGGT.

MMP13 activity assay

For measurement of MMP13 enzymatic activity, the SensoLyte® Plus 520 MMP-13 Assay Kit (AnaSpec, AS-72019) was used according to the manufacturer's instructions with 100 μl non-concentrated media collected from non-targeted or MMP13 targeted cells.

Spheroid culture

Cells from monolayer culture at P1 and P2 were trypsinized and serum-inactivated according to standard procedures and resuspended at a concentration of 1.1×10^5 cells/ml in DMEM containing 10% FBS. 100 μl of the suspension was pipetted into multiples wells of an ultra-low attachment round bottom 96 well plate (Corning, #7007). The plate was spun at 220 rcf for 10 min at 4°C and returned to a humidified cell culture incubator. For analysis of media, spheroids were formed in 1× DMEM supplemented with 1× ITS (without FBS).

Cell viability/proliferation assay

CCK-8 reagent (Dojindo CK04-11) was used to determine relative cell numbers of edited and non-edited cells. For this, 100 μl cell culture media of edited and non-edited cells (prior to the harvest of the endpoint analysis) was pipetted into 96 well plates in triplicates and 10 μl CCK-8 reagent was added and the plate was returned to the cell culture incubator. Media from edited and non-edited cells without the CCK-8 reagent, and media without cell layer but including CCK-8 reagent were used as negative controls. Two hours after addition of the reagent, the optical density (OD) was determined at 450 nm.

Live-dead cell assay

HACs grown in 8 well slides (ibidi, cat#80826) were washed with DPBS and 0.25 ml of Live-Dead reagent (ab115347) at 5× concentration diluted in DPBS was added to wells and imaged immediately.

Immunofluorescence detection of type II collagen

For immunofluorescence of spheroids, samples were harvested 7–10 days after spheroid formation, transferred to a 0.5 ml Eppendorf test tube, rinsed in DPBS and fixed for 30 min in 10% Formalin. Spheroids were blocked for 1 h in 100% buffered normal goat serum (Chondrex, #9066) and incubated in a 1/200 dilution of an AlexaFluor488 labelled COL2A1 antibody (Bioss, bs-10589R-A488) over night at 4°C rotating. The following day, spheroids were washed 5 times with DPBS for a total of 2 h and placed on a glass slide. ProLong™ Gold Antifade Mountant with DAPI (Thermo Fisher, P36935) was added and covered with a cover glass prior to imaging.

Statistical analysis

Data was determined to be non-normally distributed and thus a two-tailed, paired Wilcoxon test was employed. Results are summarised as median with interquartile range. Statistical significance was set at $P < 0.05$. The primary outcome measure was MMP13 enzymatic activity, measured in relative fluorescence units (RFU), and secondary outcome measures were mRNA levels of MMP13 shown as relative expression levels using the delta Ct method. The software package GraphPad Prism 7.4 was used for analysis. For a matched pair analysis, cell pools that had been edited were compared to non-edited cell pools from the same donor for each comparison. We only used cell pools that showed editing efficiency of at least 50% as judged by T7E1. HACs were derived from both healthy and osteoarthritic cartilage. The donors for each experimental Figure were different. Numerical summaries of all statistical comparisons can be found in Supplementary material Table S1.

Results

CRISPR-Cas9 editing strategy

Our CRISPR-Cas9 genome editing strategy for HACs is outlined in Fig. 1. Our approach has high feasibility, and potential clinical application, being rapid, and importantly, not requiring single-cell dilution and expansion that are part of conventional approaches. As proof of principle, we show that CRISPR-Cas9 can be employed to achieve significant reduction of critical cartilage matrix degrading enzyme MMP13 in human chondrocytes.

RNP uptake and cell viability

In order to assess efficiency (and potential toxicity) of Cas9-mediated ribonucleoprotein (RNP) transfection in HACs, cells at passage 1 were transfected with RNP complexes containing Cas9 protein fused to 3 nuclear localization signals (Cas9-3NLS) and a crispr RNA (crRNA) targeting MMP13 in exon 2 (less than 200 bp after the transcriptional start site) using Lipofectamine CRISPR Max transfection reagent. Fig. 2(A)–(D) shows a fluorescent Live-Dead Assay 24 h post transfection comparing untreated (2A, B; no lipofectamine or RNPs) and transfected cells (2C,D; transfection reagent with RNP complexes). As can be seen, RNP containing lipocomplexes did not negatively affect cell viability. Transfection efficiency was determined by incorporating a fluorescently labelled tracrRNA as part of the RNP and monitoring uptake after 6 h [Fig. 2(E), (F)]. In addition, nuclear fractions were analysed at two different time points to determine presence and turnover of Cas9 protein. Fig. 2(G) shows that Cas9 protein can be detected within 3 h post-transfection, but is significantly decreased after 20 h. This is in agreement with observations of rapid turnover of the Cas9 protein after RNP transfection when compared to constitutive expression from a plasmid.

Targeting MMP13 results in efficient editing with no detectable off-targets

One of the major caveats of CRISPR-Cas9 editing is potential off-target effects that arise when Cas9 cuts at genomic loci other than the intended site due to sequence similarity of the crRNA. In preliminary experiments it was determined that maximum editing can be observed after 2 days (with about a 50% increase between 24 and 48 h, see Fig. S1). In order to determine possible off-target effects as well as on-target (MMP13) efficiency, cells were harvested 2 days after transfection and a T7E1 endonuclease cleavage assay was performed. As can be seen in Fig. 3(A), editing at the target locus MMP13 in three different donors is reproducibly achieved to at

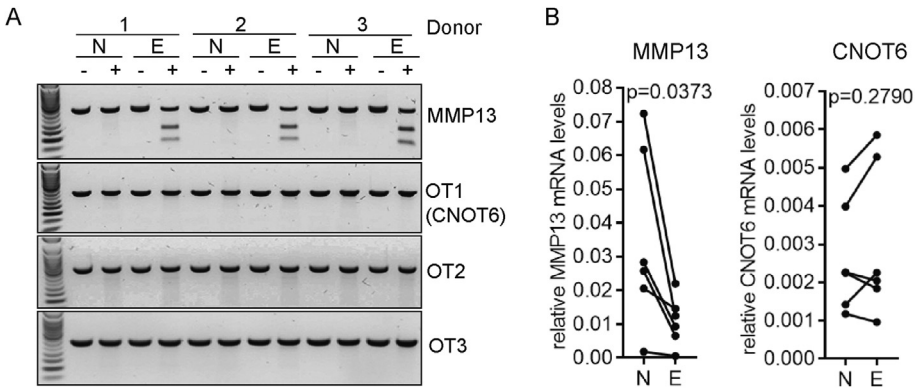

Fig. 3. Efficient editing in HACs with no off-target edits detected. A, T7E1 assay for the target area surrounding MMP13 exon2 as well as of three predicted off-targets (two intergenic and one exonic site) was performed 48 h post transfection. One OA and two healthy donors were used. B, measurement of mRNA levels of MMP13 and a protein coding predicted off-target, CNOT6 by real time qPCR. The y-axis shows relative mRNA levels normalized to RPLP0 using the delta Ct method. A paired, two-tailed Wilcoxon test was employed for statistical analysis showing the median with interquartile range. Statistical significance was set to $P < 0.05$. Samples from non-edited and edited cell populations were compared. Four OA and two healthy donors were used. OT, off-target; N, non-edited; E, MMP13-edited; −/+, without or with T7E1.

least 50%. For identification of putative off-targets, the webpage https://www.sanger.ac.uk/htgt/wge/find_off_targets_by_seq was used. No off-target activity was detected at 17 putative off-target loci in all three patients assessed. Three of these putative off-targets are shown in Fig. 3(A), the remaining can be found in supplementary material (Fig. S2).

Interestingly, there was a marked decrease in mRNA levels for MMP13 in edited cell populations averaging by about 70% as compared to control edited cell populations [Fig. 3(B)]. This reduction in mRNA levels was not observed for CNOT6, a protein coding putative off-target of this crRNA, indicating that the reduction in mRNA levels observed for MMP13 was due to the editing event.

Edited cell populations retain their edits after subsequent passaging and display greatly reduced MMP13 protein levels and activity

To investigate whether Cas9-modified chondrocytes maintain their edits and proliferate normally, transfected cells were subsequently passaged 2 days after transfection (TF), and allowed to grow to confluence with media changes every 3 days for 12–14 days. A T7E1 was performed to demonstrate maintenance of edits between 2-day post TF and the experimental endpoint 12–14 days after splitting [Fig. 4(A)]. At the same time, we confirmed by western blot that Cas9 protein was present initially 3 h post-TF but cleared from the cell pool of transfected cells after 2 days and was likewise absent 12–14 days after splitting/expansion [Fig. 4(B)]. We did not observe a compensatory effect by another major collagenase, MMP1, upon targeting and reduction of MMP13 (Fig. S3).

For our approach to be functionally useful for cell-based cartilage repair therapies, we needed to determine whether the editing efficiency that was achieved would result in significant reduction of MMP13. In monolayer cell culture, MMP13 is secreted into the media at very low levels and its expression is greatly upregulated upon treatment with IL-1β, one of the major pro-inflammatory cytokines. Non-edited and edited cell populations were induced with IL-1β at 0.1 ng/ml 20–24 h post induction, media from cells were harvested and analysed by western blot. Indeed, we found that MMP13 levels in edited cell populations were dramatically reduced (compared to non-edited) in IL-1β treated cultures [Fig. 4(C)]. The same media was analysed for enzymatic activity using a fluorimetric peptide cleavage assay specific for MMP13. The assay confirmed that total MMP13 enzymatic activity of edited cell populations was greatly reduced [Fig. 4(D)]. Due to increased sensitivity of this assay as compared to western blot analysis, a statistically significant reduction of enzymatic activity was also observed without IL-1β treatment.

In order to demonstrate that supernatant assays originated from a comparable number of cells, a cell counting assay, which measures metabolic activity of cells, was employed to compare edited and non-edited cell populations after the expansion step. As it can be seen in Fig. 4(E), there is no significant difference in cell number of both cell populations.

Thus, Cas9-editing of HACs is efficient and results in a dramatic reduction of MMP13 protein levels and subsequent enzymatic activity.

Edited loci result in a frameshift stop codon

In order to further probe the nature of the edits at the MMP13 locus, DNA regions from three donors surrounding the targeted site of MMP13 in exon2 were amplified by PCR, subcloned into a cloning vector and 24 clones for each donor were picked and sequenced in order to estimate both editing frequency (percentage of mutated clones compared to wildtype sequences) and to determine the nature of edits at the site (Table I). Editing efficiency was between 63% and 74%. Moreover, in excess of 90% of edited sequences resulted in a frameshift and premature stop codons if analysed by http://web.expasy.org/translate/indicating the majority of edited loci will result in mRNA templates containing premature STOP codons that would undergo non-sense mediated decay.

MMP13 edited cell populations show increased levels of collagen type 2 in 3D spheroid culture

Type II collagen (encoded by the COL2A1 gene) is the major collagen of the cartilage extracellular matrix and the main target of secreted MMP13. To further investigate MMP13 editing effects, a 3D culture model (spheroids) was used as secreted proteins are better retained than in simple 2D monolayer cultures. Initially, spheroids were probed for collagen-deposition by digestion with collagenase A and staining with picrosirius red (Supplementary Fig. S4). Spheroids of non-edited and edited cells were grown for up to 10 days and assayed for MMP13 and COL2A1. Importantly, MMP13 expression can be easily detected by western blot analysis without the need of IL-1β induction and is significantly reduced in media from MMP13-edited cells [Fig. 5(A)]. Protein levels of major cartilage component type II collagen were investigated by western blot [Fig. 5(B)] and immunofluorescence [Fig. 5(C)] and demonstrate increased type II collagen protein levels in MMP13-edited spheroids. Two representative samples out of four different experiments are shown to demonstrate COL2A1 increase.

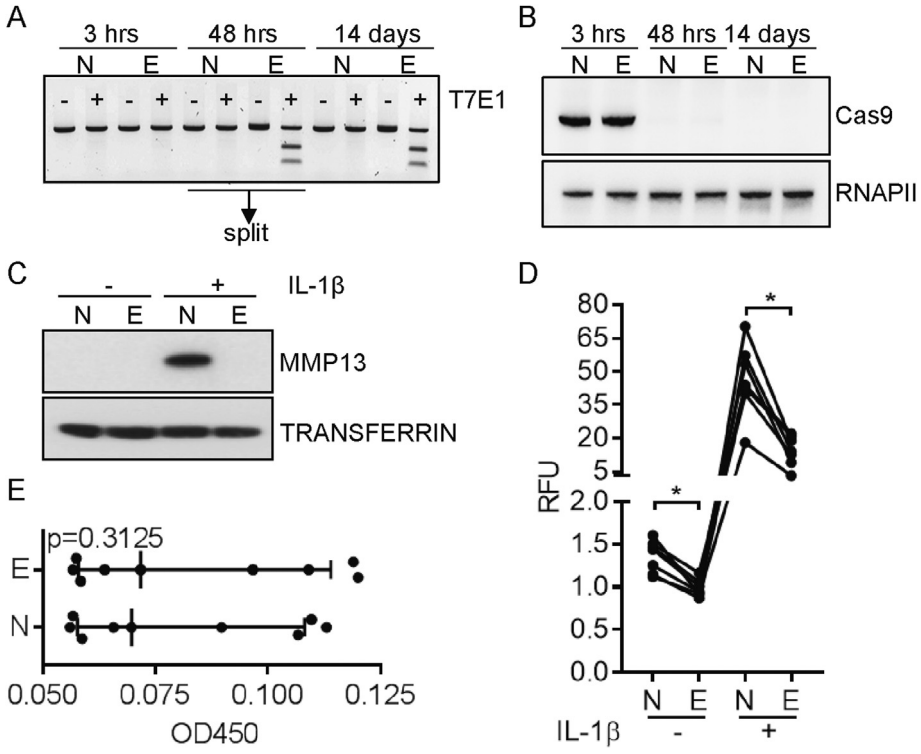

Fig. 4. Edited cell populations retain their edits after subsequent passaging and display greatly reduced MMP13 protein levels and activity. A, T7E1 assay 3 h, 48 h and 14 days post-TF. Cells were split 2 days after TF and expanded for another 12 days. B, western blot of nuclear fractions from time points used in A. C, western blot of cell culture media of edited and non-edited cell population 22 h post IL-1β induction at the experimental endpoint of 2 weeks. Different donors were used for panels A, B and C. D, MMP13 activity assay without or with IL-1β induction comparing edited and non-edited cell populations after 2 weeks. Medians ± IQR for non-edited and edited without IL-1β are 1.438 ± 0.354 for N and 0.947 ± 0.145 for E, and after IL-1β induction 43.903 ± 17.055 for N and 14.062 ± 11.922 for E. Five OA and two healthy donors were used. E, cell counting assay using CCK-8 reagent comparing edited and non-edited cells after 2 weeks. Seven OA and two healthy donors were used. A paired, two-tailed Wilcoxon test was employed for statistical analysis and showing the median with interquartile range. Samples from non-edited and edited cell populations were compared. *, $P < 0.05$; N, non-edited; E, edited; −/+, without or with IL-1β at 0.1 ng/ml; relative fluorescence units (RFU), RFU; OD450, optical density at 450 nm.

Table I
CRISPR-Cas9 editing efficiency in human articular chondrocytes (derived from one OA, and two healthy donors) and insertion/deletion characteristics at the MMP13-exon2 locus. Note: OA – osteoarthritic; H – healthy cartilage; bp – base pair; nd – none detected

Donor	1	2	3
Age (years)	68	53	25
Tissue source	OA	H	H
Editing (%)	66	63	74
Size of deletions (bp)	1–16	1–18	1–17
Size of insertions (bp)	Nd	Nd	1–3
Edited sequences resulting in premature STOP codon (%)	>90%		

This shows that editing of MMP13 by Cas9 does not only result in reduction of secretion of the targeted enzyme but also leads to increased levels of type II collagen.

Discussion

In the present study we demonstrate a CRISPR-Cas9 mediated genome editing strategy (Fig. 1) that can be used to efficiently and reproducibly establish cell populations of human articular chondrocytes (both healthy and osteoarthritic) with stably reduced expression of MMP13, a key cartilage degrading enzyme. We report editing efficiencies of 63–74% in HACs with no detectable off-target activity and we demonstrate that the resulting cell populations faithfully maintain their genome edits during subsequent expansion and culture. Three-dimensional spheroid cultures containing MMP13-edited cells showed enhanced levels of type II collagen demonstrating a chondroprotective effect. Such properties indicate this editing process can easily be incorporated into the procedures of current cell-based repair therapies such as ACI, and may enhance their efficacy.

CRISPR-Cas9 genome engineering has been widely used to create cell lines with complete ablation of a protein of interest in order to study its downstream effects[18–22]. In order to achieve this, single cell expansion of positively edited cells (i.e., cells that have a biallelic occurrence of an indel resulting in a premature stop codon in the open reading frame) is necessary. In the current approach, we use a different strategy (outlined in Fig. 1) that is potentially better adapted for clinical application: rather than selecting for biallelically edited cells we aimed to reduce the total amount of secreted MMP13 by using CRISPR-Cas9 to achieve editing of 63–74% of alleles in a given chondrocyte population. This leads to controlled reduction, rather than complete elimination, of the amount of collagen degrading MMP13 secreted into the extracellular cartilage matrix. We chose this approach for two main reasons: firstly, HACs cannot be expanded as single cells making the single-cell-to-colony approach technically not feasible; secondly, MMPs are mostly known in the context of disease, they do however have important functions in maintaining a healthy cartilage matrix (reviewed in[23]) and thus should not be completely removed from the cellular proteome. The strategy adopted here underlines the power of CRISPR-Cas9, even without selection, and we argue that reduction rather than removal of an important protein of interest is a biologically more relevant (and potentially less toxic) approach. Alternatively, CRISPRi approaches by lentiviral transduction hold

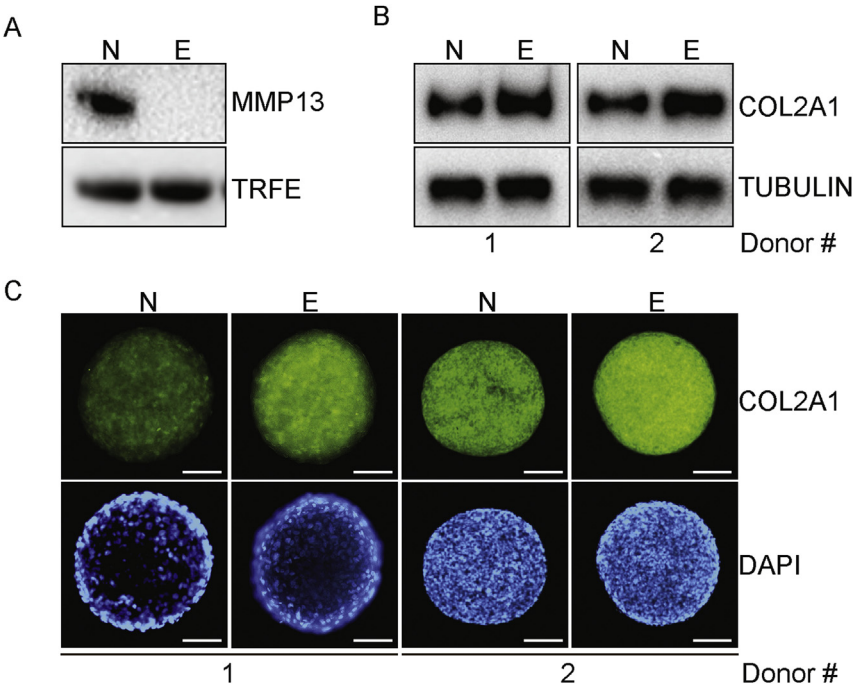

Fig. 5. MMP13 editing results in increased in type II collagen accumulation in spheroid HAC cultures. A, Cell culture media of non-edited and edited cells 7 days post spheroid formation was harvested and analysed by western blot. B, immunoblot for type II collagen (COL2A1) from spheroids of edited and non-edited cells harvested 7 days post spheroid formation. C, immunofluorescence for COL2A1 comparing non-edited and edited spheroids. Scale bar = 100 μm. N, non-edited; E, MMP13-edited; TRFE, transferrin.

promise for potent transcriptional repression[24,25]; they do however bear the potential danger of undesirable, oncogenic integration of the viral DNA into the human genome[26,27].

A potential drawback of Cas9 mediated genome engineering are off-target effects which occur when Cas9 cuts at places other than the intended target locus[28]. In order to minimize the possibility, we chose transfection of Cas9 RNP that have been shown to cause less off-target effects in comparison to plasmid based approaches. This is mostly due to the shortened half-live of Cas9 protein within the RNP to less than 12 h as compared to constitutive expression from a vector[29]. Additionally, keeping in mind potential clinical application, introduction of a double stranded plasmid would be undesirable since random integration of the plasmid DNA in the individual's genome cannot be excluded. Furthermore, for an off-target effect to be truly hazardous it would have to occur in a significant genomic locus – either within the coding region of an essential gene or within an enhancer/repressor element that creates a malignancy. Notably, there are now Cas9 enzymes available that have been modified in their DNA-binding domain to minimize occurrence of possible off target cutting and at the same time retaining their on-target activity[30]. We detected no off-target activity in the current study, however, we acknowledge that, despite recent advances[31] no current method is able to fully confirm the absence of any unwanted editing in any single cell after the editing step. We argue that a tissue like cartilage is a relatively safe harbour since edited cells are much less likely to migrate out of the cartilage due to its lack of vascularization and the minimal proliferative activity of chondrocytes within the matrix. Nevertheless, genome-wide screening for off-target activity would be essential before clinical application of CRISPR-Cas9 for cartilage cell-based therapies.

Cas9 mediated DNA editing at the MMP13 locus also leads to decrease in levels of MMP13 mRNA. While the initial, marked drop 48 h post transfection can be attributed to a combination of direct interference between Cas9 and the RNA polymerase II complex as well as degradation of the faulty, premature STOP codons containing mRNA template, the residual reduction in mRNA visible after 2 weeks (Supplementary Fig. S5) may be mostly caused by the latter mechanism.

The use of spheroids as three-dimensional tissue models has previously been shown to more closely mimic the natural tissue environment for a variety of cell and tissue types[32–34]. Interestingly, unlike monolayer culture, MMP13 protein levels in spheroids obtained from HACs were easily detected without the need for cytokine (IL-1β) induction. This is a major advantage since IL-1β treatment of HACs leads to a reduction of COL2A1 levels on the transcriptional level[35,36] and would thus confound a positive readout due to Cas9 mediated editing. Employing this model we successfully demonstrated both reduced secretion and activity of MMP13, as well as increased levels of type II within spheroids from MMP13 edited cell populations.

In summary, we demonstrate successful and significant Cas9-mediated editing of HACs without the necessity for derivation of single-cell derived clones. We believe the application of such an approach will greatly increase the efficacy of current cell-based cartilage repair therapies.

Contributions

Christine I Seidl: Conception and design, Analysis and interpretation of the data, Drafting of the article, Final approval of the article.

Tudor A. Fulga: Analysis and interpretation of data, Obtaining of funding, Final approval of the article.

Chris L Murphy: Conception and design, Drafting of the article, Final approval of the article, Obtaining of funding.

Conflict of interest

None declared.

Funding sources
The project was funded by Arthritis UK grant number 20902.

Acknowledgements

We thank Dr. Paula Dhiman (Centre for Statistics in Medicine, NDORMS, Oxford University, UK) for help and advice with statistical analysis.

Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.joca.2018.09.001.

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