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The osteogenic and angiogenic potential of microRNA-26a delivered *via* a non-viral delivery peptide for bone repair

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ABSTRACT

Bone-related injuries and diseases are among the most common causes of morbidity worldwide. Current bone-regenerative strategies such as auto- and allografts are invasive by nature, with adverse effects such as pain, infection and donor site morbidity. MicroRNA (miRNA) gene therapy has emerged as a promising area of research, with miRNAs capable of regulating multiple gene pathways simultaneously through the repression of post-transcriptional mRNAs. miR-26a is a key regulator of osteogenesis and has been found to be upregulated following bone injury, where it induces osteodifferentiation of mesenchymal stem cells (MSCs) and facilitates bone formation. This study demonstrates, for the first time, that the amphipathic, cell-penetrating peptide RALA can efficiently deliver miR-26a to MSCs *in vitro* to regulate osteogenic signaling. Transfection with miR-26a significantly increased expression of osteogenic and angiogenic markers at both gene and protein level. Using a rat calvarial defect model with a critical size defect, RALA/miR-26a NPs were delivered *via* an injectable, thermo-responsive Cs-g-PNIPAAm hydrogel to assess the impact on both rate and quality of bone healing. Critical defects treated with the RALA/miR-26a nanoparticles (NPs) had significantly increased bone volume and bone mineral density at 8 weeks, with increased blood vessel formation and mechanical properties. This study highlights the utility of RALA to deliver miR-26a for the purpose of bone healing within an injectable biomaterial, warranting further investigation of dose-related efficacy of the therapeutic across a range of *in vivo* models.

1. Introduction

Bone osteogenesis is a complex process that is closely regulated by signaling pathways of both committed and non-committed osteoprogenitor cells, resulting from the chemical stimuli of growth factors, cytokines and transcription factors [1]. MicroRNAs (miRNAs) regulate gene expression on a post-transcription level by binding to the target sequences in the 3′ untranslated region (UTR) of one or more messenger RNA (mRNA) molecules [2]. This interaction results in translational arrest or degradation by the RNA-induced silencing complex, leading to repressed production of cellular proteins. Inhibition of mRNA translation by miRNAs has emerged as an important regulator of developmental osteogenic signaling pathways, osteoblast growth and differentiation, osteoclast-mediated bone resorption activity, and bone homeostasis in the adult skeleton. Although miRNAs have a relatively fast decay, the regulation of existing mRNA and subsequent proteins can have longer-term effects [3]. Consequently, miRNAs can provide temporary, rapid tuning of gene expression. They are also involved in

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osteobflast dfifferentfiatfion of mesenchymafl stem cflfls (MSCs) through the reguflatfion of Notch, transformfing growth factor-β (TGF-β), bone morphogenetfic protefin (BMP), tumour necrosfis factor aflpha (TNF-α), and Wnt/β-catenfin sfignaflflfing pathways. Thfis mfiRNA can efither enhance or finhfibfit dfifferentfiatfion by bfindfing to osteogenfic transcrfiptfion factors that fincflude gflycogen synthase kfinase-3 beta (GSK3β), Notch2, runt-Reflated transcrfiptfion factor 2 (RUNX2), and transformfing growth factor beta (TGF-β) [4–6]. Furthermore, mfiRNAs have been fidentfiffied as reguflators of osteobflast and osteocflast behavfiour, and are finvoflved fin the process of bone remodeflflfing and mufltfipfle bone metaboflfic dfiseases such as osteoporosfis [7].

Some mfiRNAs are downreguflated durfing normafl osteogenesfis and, as a resuflt, are consfidered as osteogenfic finhfibfitors (mfiR-138, [8] 141, [9] 338 [10]); others are upreguflated and are consfidered osteogenfic promoters (mfiR-29b, [11] 196a, [12] 378 [13]). The flfiterature on a seflectfion of these mfiRNAs has reveafled contradfictory resuflts, whfich suggests that a adeeper understandfing of the bfioflogficafl cues fis requfired. For exampfle, mfiR-29a can efither finhfibfit or promote osteogenesfis dependfing on the experfimentafl condfitfions [14,15]. Thfis exempflfiffies the finherent compflexfity of controflflfing severafl gene reguflatory networks sfimufltaneousfly, where there can be extensfive crosstafllk of the moflecuflar mechanfisms finvoflved fin osteogenfic dfifferentfiatfion. Therefore, fit fis crfitficafl that afll unfintended bfioflogficafl consequences are consfidered when a mfiR therapy fis seflected for use fin human patfients.

The reguflatory rofle of mfiR-26a fin osteogenesfis has been an area of much debate. Conffflictfing reports have been pubflfished on whether overexpressfion of mfiR-26a finduces osteogenesfis-finhfibfitfing or osteogenesfis-promotfing effects. For exampfle, Luzfi, et afl. concfluded that the presence of mfiR-26a had an finhfibfitory effect on the osteogenfic dfifferentfiatfion of human adfipose-derfived stem cflfls (hADSCs). Conversefly, finhfibfitfion of mfiR-26a by 2′-*O*-methyfl-antfisense RNA fincreased protefin flevefls of SMAD1 transcrfiptfion factor wfith enhanced osteodfifferentfiatfion [16]. Lfikewfise, Lfi, et afl. reported that overexpressfion of mfiR-26a finhfibfited osteodfifferentfiatfion of mouse ADSCs *vfia* dfirect targetfing of the 3′UTR of Wnt5a, and subsequentfly downreguflated the Wnt/Ca^{2+} sfignaflflfing pathway [17].

However, mfiR-26a has afso been reported to target GSK3β to actfivate the Wnt/β-catenfin-sfignaflflfing pathway whfich transflocates β-catenfin from the cytopflasm to the nucfleus, thus enhancfing fits transcrfiptfion. [18,19]. GSK3β fis a negatfive reguflator of osteogenesfis *vfia* the attenuatfion of RUNX2 sfignaflflfing [20]. MfiR-26a bfinds to, and finhfibfits, GSK3β to enhance osteogenesfis through upreguflatfion of RUNX2, the master reguflator of osteobflastogenesfis [21]. MfiR-26a has afso been shown to downreguflate PTEN, whfich can flead to osteogenesfis and fimproved vascuflarfisatfion [22,23]. Specfifficaflfly, when deflfivered *fin vfitro*, mfiR-26a reguflates osteobflast functfion and promotes dfifferentfiatfion of MSCs finto osteobflasts, supportfing the use of thfis mfiRNA as a gene therapeutfic for bone regeneratfion [22,24].

Reported target genes of mfiR-26a fincflude SMAD1, GSK3β and transducer of ERBB2 1 (Tob1); these moduflate the expressfion of SMADs whfich are fintraceflfluflar protefins responsfibfle for the sfignafl transductfion of the extraceflfluflar, transformfing growth factor, beta (TGF-β) superfamfifly of flfigands [25–27]. The TGF-β famfifly of cytokfines fincfludes BMPs and growth dfifferentfiatfion factors (GDFs) such as GDF-2 and TGF-βs protefin (TGF-β1, TGF-β2, and TGF-β3). These have a fundamentafl rofle fin coupflfing bone resorptfion and bone formatfion durfing post-natafl bone homeostasfis by bfindfing to a ceflfl-membrane bound type-II receptor, and by the subsequent recrufitment and phosphoryflatfion of a type-I receptor, whfich fin turn phosphoryflates receptor-reguflated SMADS (R-SMADS) [28–30]. Therefore, mfiR-26a contrfibutes to supportfing osteogenesfis and bone formatfion by targetfing SMAD1-BMP sfignaflflfing fin bone-formfing ceflfls. mfiR-26a afso pflays a key rofle fin vascuflar endotheflfiafl growth factor (VEGF)-medfiated neovascuflarfisatfion through dfirect targetfing of Nogo-B receptor [31]. *In vfivo* deflfivery of human bone MSCs (hBMSCs), transfected wfith 50 nM mfiR-26a mfimfic from a bfiodegradabfle heparfin-hyafluronan-pofly(ethyflene gflycofl) dfiacryflate hydrogefl, facfiflfitated bone regeneratfion afong wfith fincreased vascuflarfisatfion fin a 5 mm caflvarfiafl mouse defect through osteogenesfis-angfiogenesfis coupflfing [32]. Stfimuflatfion of the finvasfive network of bflood vessefls *vfia* angfiogenesfis fis necessary for the conversfion of hypertrophfic cartfiflage tempflate to bone durfing endochondrafl bone formatfion [33]. Addfitfionaflfly, sufffficfient angfiogenesfis, often controflfled through VEGF sfignaflflfing, fis essentfiafl for adequate heaflfing of fractures [34]. Osteogenfic-angfiogenfic coupflfing fis a commonfly used approach for bone regeneratfion, and overcomfing finadequacfies fin these sfignaflflfing pathways through deflfivery of mfiR-26a may be an effectfive approach to fimprovfing bone heaflfing [35].

MfiR-26a fis afso sfignfficantfly downreguflated fin fractures. Zou, et afl. reported flevefls of mfiR-26a to be ffive tfimes flower fin patfients wfith fractures compared to heaflthy voflunteers [22]. Overexpressfion of mfiR-26a fleads to: finhfibfitfion of osteobflast apoptosfis and decrease of cflfl death; promotfion of osteobflastfic actfivfity; formatfion of mfineraflfised nodufles through an fincrease fin BMP-2 and WNT3a; and suppressfion of osteocflast formatfion by targetfing connectfive tfissue growth factor (CTGF) [22,36]. Weekfly deflfivery of 15 μg of mfiR-26a mfimfic, finjected finto the fracture sfite of femur perfiosteum for 8 weeks, promoted heaflfing of a non-unfion fracture fin a rat modefl by targetfing SOSTDC1 and Wnt/β-catenfin sfignaflflfing pathways [37]. Thfis further supports cflafims that over-expressfion of mfiR-26a coufld promote osteogenesfis.

Sfince the effficfiency of any nucflefic acfid therapy fis dependent on fintraceflfluflar transport, a deflfivery system fis requfired that protects the cargo from degradatfion, crosses the cflfl membrane, and escapes the endosome wfithout evokfing an fimmune response [38]. The amphfipathfic fusogenfic peptfide RALA has been shown to compflex anfionfic cargoes firrespectfive of sfize, protect the cargo from degradatfion, and escape endosomafl envfironments ensurfing reflease of the cargo finto the cytopflasm [39–48]. Thfis study reports for the ffirst tfime on the use of RALA to deflfiver mfiR26a, fin both DNA and mfiR mfimfic forms, from a thermoresponsfive hydrogefl for bone regeneratfion [49]. Studfies were specfifficafflfly desfigned to examfine the physfiochemficafl characterfistfics of the RALA/mfiR-26a nanopartficfles, the osteogenfic potentfiafl and gene targets fin hBMSCs, the degradatfion proffifle of the hydrogefl and the osteogenfic and angfiogenfic potentfiafl fin a rat crfitficafl-sfized caflvarfiafl defect modefl.

2. Materials and methods

2.1. Materfiafls

2.1.1. The RALA peptfide

The RALA peptfide was produced by sofffid state synthesfis (ffluorenyflmethoxycarbonyfl chemfistry) (Bfiomatfik, Canada) and suppflfied as a flyophfiflfised powder that requfired reconstfitutfion before use. Peptfides were suppflfied fin the acetate safft form and > 95% purfity. Foflflowfing cfleavage from the resfin, the desfired products were purfiffied and conffirmed by Reverse Phase Hfigh Performance Lfiqufid Chromatography (HPLC) usfing a 1260 Inffinfity II LC System (Agfiflent, USA).

2.1.2. Nucflefic acfids

pEGFP-N1 (Cflontech, USA) and pcDNA3-mfiR26a2 (Addgene, UK) pflasmfids were purchased and subsequentfly propagated usfing MAX Effficfiency® DH5α™ Competent Cflfls (Lfife Technoflogfies, UK). Purfifficatfion was compfleted usfing a PureLfink® HfiPure Pflasmfid Ffiflter Maxfiprep Kfit (Lfife Technoflogfies, UK) and quantffified by UV absorptfion at 260 nm usfing a Nanodrop (Thermo Ffisher Scfientfiffic, UK). For the *fin vfivo* studfies, a doubfle-stranded mfiR-26a-5p mfimfic was deflfivered (Dharmacon, UK).

2.1.3. Cflfl flfines

Human bone marrow-derfived mesenchymafl stem cflfls (hBMSC) (Promoceflfl, Germany) were used. Proffiflfing of the cflfl flfine was compfleted by the suppflfiers and was conffirmed as *Mycopflasma*-free by monthfly testfing usfing the MycoAflert® Mycopflasma Detectfion Kfit (Lonza, Swfitzerfland).

2.1.4. Anfimafls

Nfine C57/BL6 mfice used fin the *fin vfivo* degradatfion study were housed fin an open facfiflfity fin Queen's Unfiversfity Beflfast and experfimentafl protocofls were compflfiant wfith the UK Scfientfiffic Act 1986. Twenty-efight Wfistar mafle rats used fin the caflvarfiafl defect study were housed fin the temperature-controflfled anfimafl care facfiflfity at the Unfiversfity of Massachusetts Amherst (USA). Affl housfing and *fin vfivo* experfimentafl procedures were performed fin accordance wfith protocofl #1815 approved by the Instfitutfionafl Anfimafl Care and Use Commfittee (IACUC) of the Unfiversfity of Massachusetts Amherst. Anfimafls had *ad ffifibfitum* access to food and water durfing the experfiment and were kept under standard day/nfight cycfle.

2.2. Methods

2.2.1. mfiR-26a gene target predfictfion

In sfiffico anaflysfis of four pubflficfly avafiflabfle bfiofinformatfics aflgorfithms – mfiRDB, TargetScan, mfiRTarBase and mficrocosm, [50,51] – were compfleted to fidentfify the gene targets fin whfich mfiR-26a fis predficted to bfind to the 3′ UTR. The data were coflflated and arranged finto a four-way Venn dfiagram. Target genes seflected for further anaflysfis, *fi.e.* coflflagen Type 1 (COL1), RUNX2, osteocaflcfin (OCN), and vascuflar endotheflfiafl growth factor A (VEGF-A), were based on finformatfion avafiflabfle fin the flfiterature wfith regards to bone regeneratfion fin hMSCs.

2.2.2. Synthesfis of NPs

RALA/pmfiR-26a and pDNA nanopartficfles (NPs) seflf-assembfle *vfia* eflectrostatfic finteractfions under ambfient temperatures. RALA/pDNA NPs were prepared at N:P ratfios 1–15 by addfing approprfiate voflumes of RALA peptfide soflutfion to 1 μg pDNA as prevfiousfly descrfibed [39]. The NPs were fincubated at ambfient temperature for 30 mfin to afflow the formatfion of the compflexes. RALA/mfiR26a mfimfic NPs at N:P ratfio of 8 were chosen for *fin vfivo* anaflysfis (optfimfisatfion data not shown).

2.2.3. NPs sfize and zeta potentfiafl anaflysfis

The mean hydrodynamfic sfize of nanocompflexes synthesfised usfing 1 μg of pmfiR-26a dfispersed fin 50 μL of H_2O was determfined usfing Dynamfic Lfight Scatterfing at 20 °C wfith a Maflvern Nano ZS (Maflvern Instruments, UK). Mean surface charge (zeta potentfiafl) of the compflexes was aflso measured by usfing Laser Doppfler Veflocfimetry on the Maflvern Nano ZS system.

2.2.4. Encapsuflatfion effficfiency of mfiR-26a wfith the RALA peptfide

mfiR-26a NPs (1 μg of pmfiR-26a) were formuflated at varfious N:P ratfios and added fin trfipflficate to a bflack 96-weflfl mficropflate (Thermo Ffisher Scfientfiffic, UK). 50 μL of Quant-fiT™PficoGreen® Reagent (Lfife Technoflogfies, UK) dfifluted 1:200 fin TAE 1× buffer was subsequentfly added to each weflfl and fincubated for 30 mfin at RT. Sampfle ffluorescence was measured at 480/520 nm usfing a FLUOstar Omega mficropflate reader (BMG Labtech, Germany). Encapsuflatfion effficfiency was reported reflatfive to a pmfiR-26a-onfly controfl. Data are reported as the mean of three findependent repeats ($n = 3$, ± SEM).

The compflexatfion of pDNA by the RALA peptfide was conffirmed usfing Ion Exchange Chromatography (IEC). 0.5 g of the Ion Exchange Medfia, SP-Sephadex (Sfigma-Afldrfich, SPC25120, GER), was fincubated fin 10 mL of 1 M moflecuflar bfioflogy grade NaCfl (Sfigma-Afldrfich, Germany) overnfight at room temperature to facfiflfitate swefflfing of the coflumn. Foflflowfing supernatant removafl, the resfin was washed three tfimes usfing 10 mL Ufltrapure water to ensure no fionfic soflvent was present. 2 mL of washed resfin was floaded finto a frit coflumn and packed under pressure. 20 μL of pmfiR-26a or RALA/pmfiR-26a NPs at ≥20 mg/mL concentratfion was floaded finto the coflumn and efluted wfith 3 mL of Ufltrapure water. Ffinafllfly, fractfions were coflflected fin 0.5 mL centrfifuge tubes and anaflysed usfing a Nanodrop 2000 (Thermo Scfientfiffic, UK).

2.2.5. Transfectfion studfies fin human BMSCs

hBMSCs were seeded at a densfity of 30 × 10^3 ceflfls finto 24-weflfl pflates (Thermo Scfientfiffic, USA) 24 h prfior to treatment. Once confffluent, ceflfls were condfitfioned for 2 h fin Optfi-MEM reduced serum medfia (Gfibco, UK). Weflfls of the mufltfi-dfish were then suppflemented wfith a treatment of RALA/pmfiR-26a NPs, such that the totafl pmfiR-26a fin the weflfl was 3.3 μg/cm², and RALA was 26.1 μg/cm². The foflflowfing treatments were added to weflfls for comparatfive studfies: fi) untreated controfl, ffi) RALA controfl (RALA/pEGFP-N1), wfith equfivafient RALA used and fffi) RALA/pmfiR-26a NPs. Foflflowfing fincubatfion for 4 h under standard cuflture condfitfions, the medfia was removed and repflaced wfith serum-suppflemented (10% FBS, 1% Pen/Strep) standard hMSC expansfion medfia (XPAN). Ceflfluflar response was verfiffied foflflowfing post-transfectfion at weekfly fintervafls. Osteogenfic markers and predficted mfiR-26a targets were anaflysed by reverse transcrfiptfion poflymerase chafin reactfion (RT-PCR), hBMSCs were suppflemented wfith osteofinductfion medfia (Promocefl, Germany) 72 h post-transfectfion, and a further negatfive controfl group of ceflfls added that was cufltured fin standard growth medfia (no osteogenfic finductfion) to determfine prfimer specfifficfity.

2.2.6. Quantfitatfive Reafl-Tfime PCR (qRT-PCR) for mfiR-26a expressfion from human BMSCs

RNA was extracted from hBMSCs *vfia* phase separatfion usfing a *mfir*-Vana fisoflatfion kfit (Thermo Ffisher Scfientfiffic, UK). Reverse transcrfiptfion reactfions for mfiR-26a and the endogenous controfl U6 spflficeosomafl RNA (U6) were compfleted accordfing to the manufacturer's protocofl (TaqMan Smaflfl RNA assay, Invfitrogen, UK). Quantfitatfive reverse transcrfiptfion poflymerase chafin reactfion (qRT-PCR) of mfiR-26a flevefls was performed usfing a Lfightcycfler 480 II (Roche, UK) accordfing to the foflflowfing parameters: 95 °C for 10 mfin (pre-fincubatfion), then ampflfiffficatfion *vfia* 40 cycfles of 95 °C for 15 s, and 60 °C for 60 s. mfiR-26a expressfion was determfined from the recorded Ct vaflues usfing the ΔΔCT method, wfith gene expressfion reported as fofld change compared to an untreated controfl, wfith U6 as the endogenous controfl.

2.2.7. Fflow cytometrfic anaflysfis for GFP expressfion

48 h post-transfectfion, hBMSCs transfected wfith RALA/pEGFP-N1 NPs were anaflysed for the presence of green ffluorescent protefin (GFP). GFP-expressfing hBMSCs were detected usfing the ffluorescence-actfivated ceflfl sortfing (FACS) caflfibur system (BD Bfioscfiences, UK), and data were anaflysed usfing BD CeflflQuest™ Pro software. Resuflts are reported as the mean of three findependent repeats ($n = 3$, ± SEM).

2.2.8. qRT-PCR anaflysfis of mfiR-26a target genes

For each of the target genes – COL1, RUNX2, OCN and VEGF-A – a specfiffic prfimer set (forward and reverse, Tabfle 1) was obtafined from Integrated DNA Technoflogfies, UK. qRT-PCR anaflysfis was performed usfing a Lfightcycfler 480 II (Roche, UK) wfith the foflflowfing flfight cycflfing parameters: 95 °C for 5 mfin (pre-fincubatfion), then ampflfiffficatfion *vfia* 45 cycfles of 95 °C for 10 s, 60 °C for 10 s, and 72 °C for 10 s. Gene expressfion was determfined from the recorded Ct vaflues usfing the ΔΔCT method, wfith gene expressfion reported as fofld change compared to an untreated controfl, wfith gflycerafldehyde-3-phosphate dehydrogenase (GAPDH) as an endogenous controfl.

2.2.9. Immunoffluorescence mficroscopy for flate stage osteogenfic markers fin hBMSCs

hBMSCs were pflated on 13 mm coversflfips (Agar Scfientfiffic, UK) and pflaced fin a 24-weflfl pflate at a densfity of 30 × 10^3 ceflfls per weflfl. Foflflowfing transfectfion wfith mfiR-26a NPs and cuflture fin standard hMSC growth medfia (Promocefl, Germany), ceflfls were ffixed usfing 4% paraformafldehyde (Sfigma-Afldrfich, UK) for 30 mfin at RT, washed wfith PBS, and bflocked for 1 h usfing PBS contafinfing 1% bovfine serum aflbumfin (Sfigma-Afldrfich, UK) and 0.01% Tween 20 (Sfigma-Afldrfich, UK). Ceflfls were next fincubated for 1 h at 37 °C wfith antfi-osteocaflcfin (1:100, sc-

Table 1
Gene specifific prfimer sets (Forward and Reverse).

Gene Target (NCBI Reference)	Descrfiptfion	Prfimer Sequence	
COL1A1 (NM_000088.3)	*Homo sapfiens* coflfagen type I aflpha 1 chafin	FWD	5′ AAG AAC CCC AAG GAC AAG AG 3′
		REV	5′ GTA GGT GAT GTT CTG GGA GG 3′
BGLAP (OCN) (NM_199173)	*Homo sapfiens* bone gamma-carboxygflutamate protefin (OCN)	FWD	5′ GGC AGC GAG GTA GTG AAG AG 3′
		REV	5′ CTC ACA CAC CTC CCT CCT G 3′
RUNX2 (NM_001024630.3)	*Homo sapfiens* RUNX2	FWD	5′ GCG GTG CAA ACT TTC TCC AG 3′
		REV	5′ GAC TCT GTT GGT CTC GGT GG 3′
VEGF-A (NM_001025366.2)	*Homo sapfiens* VEGF-A	FWD	5′ GAG CCT TGC GCT CTA 3′
		REV	5′ CAC CAG GGT CTC GAT TGG ATG 3′

21,742, Santa Cruz, USA) or antfi-osteopontfin (1:100, sc-73,464, Santa Cruz, USA) antfibodfies. Foflflowfing washfing fin PBS, the prfimary antfibody was fincubated for 1 h at 37 °C wfith bfiotfinyflated horse antfi-mouse Immunogflobuflfin G (IgG) secondary antfibody (1:200, BA-2000, Vector Labs, UK). Membranes were washed three x 5 mfin wfith PBS, and fincubated for 30 mfin at 4 °C wfith ffluorescefin streptavfidfin (1:200, SA-5001, Vector Labs, UK) as a ffluorescent probe. Ffinaflfly, coversflips were mounted on mficroscope sflfides (Agar Scfientfiffic, UK) wfith the use of DAPI-contafinfing mountfing medfia (ab104139, Abcam, UK) to vfisuaflfise cefl nucflefi. Ffluorescentfly stafined ceflls were observed by Immunoffluorescence (IF) mficroscopy usfing a TSC SP5-Lefica Mficrosystems confocafl mficroscope (Lefica, UK).

Images were anaflysed for normaflfised ffluorescence usfing the fimagfing software Ffjfi v 2.10.0 (an open-source extensfion of ImageJ) [52]. For cefl countfing, the 'Anaflyse Partficles' macro was used on the fimages of DAPI-stafined nucflefi of ceflls, wfith a sfize-flfimfitfing parameter of 200 pfixefls. For OCN and osteopontfin (OPN) ffluorescence fintensfity, the flevefl of background ffluorescence was set as a threshofld parameter, and the mean ffluorescence fintensfity of the fimage was determfined. Each sampfle was fimaged fin three random flocatfions per weflfl, and reported vaflues are the resuflt of three findependent experfiments.

2.2.10. In-cefl western assay for RALA/pmfiR-26a effect on hBMSCs flate-stage osteogenfic markers

hBMSCs were pflated fin a 24-weflfl pflate at a densfity of 30 × 10³ cefls per weflfl. Foflflowfing transfectfion wfith RALA/pmfiR-26a NPs and cuflture fin standard hMSC growth medfia (Promocefl, Germany), treated and controfl cefls were ffixed usfing 4% paraformafldehyde (Sfigma-Afldrfich, UK) for 30 mfin. Cefls were then permeabfiflfised at 4 °C for 4 mfin by addfitfion of 0.1% Trfiton X-100 (Sfigma-Afldrfich, UK) fin PBS. For bflockfing, PBS/1% Marvefl mfiflk powder (Asda, UK) was added to the cefls and fleft for 90 mfin on a shaker at 37 °C. After bflockfing, the cefls were washed twfice fin 1% mfiflk/PBS, and cefls were next fincubated for 2 h at 37 °C wfith antfi-osteocaflcfin (1:100, sc-21,742, Santa Cruz, USA) or antfi-osteopontfin (1:100, sc-73,464, Santa Cruz, USA) prfimary antfibodfies, aflongsfide CeflfITag 700 (LI-COR Bfioscfiences, UK) fin a 1/500 dfiflutfion fin PBS, for flater anaflysfis of the normaflfisatfion to cefl number. After prfimary antfibody fincubatfion, cefls were washed ffive tfimes for 5 mfin on a shaker wfith wash buffer (PBS and 0.1% Tween 20). Foflflowfing washfing fin PBS, the prfimary antfibody was fincubated for 1 h at 37 °C wfith bfiotfinyflated horse antfi-mouse IgG secondary antfibody (1:10,000, BA-2000, Vector Labs, UK), washed, and fincubated for 30 mfin wfith ffluorescefin streptavfidfin (1:200, SA- 5001, Vector Labs, UK) as a ffluorescent probe for the protefins of finterest. The cefls were washed, and antfibody targets were detected fin the 800-channefl (green pseudo-coflour), and normaflfised for cefl number to the antfibody sfignafls from the 700-channefl (red pseudo-coflour) usfing an Odyssey Cflassfic Infrared In-Cefl Western (ICW) Assay Imagfing System (LI-COR Bfioscfiences, UK).

2.2.11. Mfineraflfisatfion of hBMSCs: Afflfizarfin red stafinfing

To evafluate mfineraf deposfitfion fin hBMSCs transfected wfith mfiR-26a NPs, cefls were washed wfith PBS and ffixed for 30 mfin wfith 300 μL of 10% neutraf buffered formaflfin (Sfigma-Afldrfich, UK). The cefls were washed wfith dfistfiflfled water and 300 μL of 40 mM, and pH 4.2 Afflfizarfin red S (Sfigma-Afldrfich, UK) was added to the cefl monoflayer. Afflfizarfin stafinfing soflutfion was removed, and the cefls were washed wfith dfistfiflfled water to remove any unbound stafin. Stafined sampfles were afir-drfied, and mficroscopy fimages were captured usfing an EVOS XL finverted dfigfitaf mficroscope (Thermo Ffisher Scfientfiffic, UK). To quantfify the degree of ARS stafinfing, semfi-quantfitatfive anaflysfis of the ARS was performed through extractfion of the ARS dye usfing 100 mM cetyflpyrfidfinfium chflorfide (CPC) (Sfigma-Afldrfich, UK) under gentfle rockfing for 30 mfin at ambfient temperature. Ffinaflfly, the absorbance of the CPC soflutfion was read at 540 nm usfing a FLUOstar Omega mficropflate reader (BMG Labtech, Germany).

2.2.12. Fabrficatfion of Cs-g-PNIPAAm hydrogefl contafinfing RALA/mfiR-26a NPs

The bfiodegradabfle and thermosensfitfive chfitosan-grafted-pofly(N-fisopropyflacryflamfide) (Cs-g- PNIPAAm) hydrogefl used to deflfiver RALA/mfiR-26a NPs *fin vfivo* was synthesfised as prevfiousfly descrfibed [49]. Brfieffly, the Cs-g- PNIPAAm hydrogefl was synthesfised through free radficafl poflymerfisatfion by addfing NIPAAm to 1% wefight/voflume Cs fin acetfic acfid. The soflutfion was purged for 1 h usfing dry nfitrogen and poflymerfised by addfing an finfitfiator (ammonfium persuflfate, APS) and an acceflerator (*N,N,N′,N′*-tetramethyflethyflenedfiamfine, TEMED). The copoflymer was dfiaflysed for 5 days fin doubfle-dfistfiflfled water at ambfient temperature to remove any unreacted monomers and recovered by freeze-dryfing. To mfinfimfise the chances of pDNA fintegratfion finto the genome [53], a mfiR-26a mfimfic was deflfivered finstead of pmfiR-26a for the *fin vfivo* vaflfidatfion. The flyophfiflfised Cs-g-PNIPAAm copoflymer was soflubfiflfised to 5% *w/v* concentratfion wfith RALA/mfiR-26a NPs for 18 h at 4 °C. The ffinafl mfiR-26a concentratfion fin the hydrogefl used for *fin vfivo* appflficatfion was equfivaflent to 50 μg/mL. Therefore, 100 μL of each hydrogefl contafinfing 5 μg of mfiR-26a was finjected finto the caflvarfiafl defect.

2.2.13. Scannfing eflectron mficroscopy (SEM)

The flyophfiflfised hydrogefl was soflubfiflfised fin ufltrapure water +/ RALA/mfiR-26a NPs to achfieve a concentratfion of 50 μg/mL under agfitatfion at 4 °C for 18 h. SEM fimagfing was conducted usfing a Hfitachfi TM3030 wfith a vofltage of 15 kV.

2.2.14. Rheoflogy

Rheoflogficafl propertfies of the hydrogefl were tested usfing a stress-controflfled rheometer (modefl AR-2000ex) (TA Instruments, New Castfle, DE, USA) wfith paraflflefl pflate geometry (40 mm dfiameter). The reconstfituted hydrogefl wfith a voflume of 1.5 mL was used for the rheoflogficafl anaflysfis. G′ and G" vaflues were obtafined by performfing a temperature sweep at a frequency of 1 Hz over a range from 20 to 45 °C at a rate of 3 °C/mfin.

2.2.15. Hydrogefl reflease

To determfine RALA/mfiR-26a reflease, a voflume of 100 μL hydrogefl (correspondfing to the 5 μg dose used *fin vfivo*) was fincubated fin the weflfls of a 24-weflfl pflate at 37 °C for 1 h to soflfidfify. Foflflowfing thfis, 1.4 mL of

ufltrapure water was added to each of the weflfls, and the pflate was fincubated at 37 °C. At predetermfined tfimepofints, 50 μL of water was removed from each of the weflfls and stored at ambfient temperature. RALA/mfiR-26a NPs were dfissocfiated by addfing 50 μL of 0.2 mg/mL Protefinase K (Sfigma Afldrfich, UK) and fincubatfing for 1 h at 37 °C. mfiRNA concentratfion was determfined usfing a Quant-fit™ RfiboGreen RNA Assay Kfit by comparfing to a standard curve produced wfith mfiR-26a at concentratfion range of 0.1–10 ng/μL (Thermo, USA). The percentage reflease of the 5 μg mfiRNA fin each weflfl was caflculated at each tfimepofint.

mfiRNA-26a was detected from hydrogefl reflease sampfles by RT-PCR. Sampfles were coflflected and dfigested as above and 5 μL was used for reverse transcrfiptfion usfing the TaqMan™ MficroRNA Reverse Transcrfiptfion Kfit (Thermo Ffisher, UK) foflflowed by PCR usfing TaqMan™ MficroRNA Assays and TaqMan™ Unfiversafl Master Mfix II, no UNG (Thermo Ffisher, UK). PCR was conducted fin a LfightCycfler 96 (Roche, UK) and anaflysed usfing the correspondfing software.

2.2.16. Hydrogefl degradatfion

To determfine the *fin vfivo* degradatfion rate, 100 μL hydrogefl wfith or wfithout the fincorporatfion of RALA NPs was wefighed and finjected subcutaneousfly finto the fflanks of C57BL/6 mfice (N = 3 per group). Mfice were sacrfifficed at varfious tfimepofints and the hydrogefl was excfised to determfine the remafinfing mass. To sfimuflate *fin vfivo* degradatfion condfitfions, 100 μL hydrogefl was fincubated fin the weflfls of a 24-weflfl pflate fin PBS aflone or PBS contafinfing 3 mg/mL flysozyme. The pflates were fincubated for 8 weeks at 37 °C. The remafinfing mass of hydrogefl wfithfin the weflfls was determfined at varfious tfimepofints.

2.2.17. Caflvarfiafl defect surgery

Crfitficafl-sfized caflvarfiafl defect surgery was performed foflflowfing a pubflfished protocofl [54]. Wfistar rats at 12 weeks were anesthetfised *vfia* fisofflurane finhaflatfion (2.5%–3.5%). Usfing a sterfifle technfique, a 2–3 cm fincfisfion was made from the brfidge of the nose to the back of the head. The skfin and underflyfing perfiosteum were contracted flateraflfly usfing an eflevator to expose the caflvarfia. Usfing a surgficafl dfiffl and trephfine operatfing at 1500 RPM, a 6.8 mm dfiameter cfircuflar defect was made and removed fin the parfietafl bone between the temporafl flfine and the sagfittafl suture flfine. Afllthough 8 mm defects are commonfly used for crfitficafl-sfize cranfiafl defects fin rodents, dfiameters as flow as 5 mm have been used when fintroducfing two defects per anfimafl, whfich reduces the number of anfimafls needed and reduces the chance of finteractfion between adjacent defects [55–57]. Rats were randomfised finto treatment groups. For those recefivfing treatment, 100 μL voflume of hydrogefl was admfinfistered finto the defect space dfirectfly after the defect was made. After treatment, the perfiosteum was stretched back over the skuflfl finto fits orfigfinafl pflacement and sutured cflosed usfing 4/0 Maxon bfiodegradabfle sutures (Medtronfic, USA). The skfin over the perfiosteum was then sutured. The procedure ended wfith 3–6 BD autocflfips (Ffisher Scfientfiffic, USA) over the wound. Saflfine was used throughout the procedure.

Anfimafls were monfitored for sfigns of dfistress dafifly post-surgery. No fimpflant rejectfions or finfflammatory reactfions were observed fin any of the survfivfing anfimafls.

2.2.18. Mficro-computed tomography anaflysfis

Mficro-Computed Tomography (μCT) anaflysfis was performed *fin vfivo* at 4 and 8 weeks post-surgery. Anfimafls were fimaged usfing μCT (Bruker Skyscan 1276, USA) foflflowfing anesthetfisatfion wfith fisofflurane finhaflatfion. Anfimafls were scanned usfing a voxefl sfize of 40 μm and a 1 mm aflumfinfium ffifilter. The X-ray tube vofltage was 60 kV, the current was 125 μA, and the exposure tfime was 490 ms. The 3D reconstructfions were performed usfing NRecon v 2.0 (Bruker, USA). To assess bone regeneratfion fin defects, a 6.8 mm dfiameter cyflfindrficafl voflume of finterest (VOI) overflafid the defect regfion to quantfify bone voflume (mm^3) and bone mfineraf densfity (g/cm^3) of new tfissues.

2.2.19. Hfistoflogficafl anaflysfis

Foflflowfing euthanasfia *vfia* fintracardfiac exsangufinatfion at 8 weeks post-surgery, caflvarfia bones contafinfing the defect regfion were harvested (n = 3 per group), ffixed fin 10% formaflfin for 72 h, and decaflcfiffied fin formfic acfid overnfight prfior to hfistoflogficafl examfinatfion. Each tfissue sectfion was embedded fin paraffffin, and 5 μm sectfions were cut from the approxfimate mfidflfine of the defect and posfitfioned onto a gflass mficroscope sflfide. Sectfions were stafined wfith hematoxyflfin and eosfin (H&E), and fimaged at 400× magnfifficatfion usfing a Keyence BZ-X800 flfight mficroscope (Keyscence, Japan) ffitted wfith a Nfikon 40 × 0.6NA objectfive. Each fimaged sampfle was scored on a 0–3 gradfing scafle for presence of bone, bflood vessefls, and floose connectfive tfissue. A score of 0 findficated no tfissue fin the defect, and a score of 3 findficated extensfive tfissue fin the defect. Afflsampfles were graded findependentfly reflatfive to each other by two separate researchers.

2.2.20. Mechanficafl characterfisatfion

Mechanficafl characterfisatfion was compfleted to determfine the maxfimum force, compressfive strength, and the Young's moduflus, for four representatfive caflvarfiafl bone sampfles at both the defect and the naturafl bone sfites on the opposfite sfide of the parfietafl bone. Testfing was performed usfing the Zwfick Z005 testfing machfine (Zwfick/Roeflfl, Zwfick Roeflfl Ltd., UK), ffitted wfith a 500 N floadcefl. Before mechanficafl characterfisatfion, sampfles were precondfitfioned at 37 ± 0.5 °C for 1 ± 0.1 h. Testfing was repeated at three dfifferent flocatfions on the defect and the naturafl bone sfites. Mechanficafl testfing was performed fin a hydrated envfironment, usfing a PBS-ffiflfled water bath mafintafined at 37 ± 0.5 °C. A pre-floadof 0.2 N was ffirst reached before the fflat-faced cyflfindrficafl findenter (Ø1.1 mm) was used to appfly the compressfive floadfing at a rate of 2 mm/mfin, to efither a maxfimum force of 20 N or fracture. The maxfimum force (F_{Max}) was recorded for each sampfle, and the compressfive strength was caflcuflated as the quotfient of F_{Max} and the cross-sectfionafl area of the bone defect sfite. The Young's moduflus was caflcuflated from the sflope of the flfinear regfion of resufltfing stress *vs.* strafin curves.

2.2.21. Statfistficafl anaflysfis

Unfless otherwfise stated, three findependent experfiments were compfleted for each anaflysfis, and the resuflts are presented as mean ± SEM, n = 3 (where n fis equafl to the number of findependent batches prepared). Statfistficafl anaflysfis was performed usfing Prfism 6.0 (GraphPad Software, USA). Statfistficaflfly sfignfifficant dfifferences were then caflcuflated usfing the two-way anaflysfis of varfiance (2-way ANOVA), wfith a p-vaflue of ≤0.05 consfidered sfignfifficant: (*) $p \leq 0.05$; (**) $p \leq 0.01$; (***) $p \leq 0.001$.

3. Results

3.1. Identffficatfion of mfiR-26a as a potentfiafl therapeutfic target for osteogenesfis

Muflfipfle aflgorfithms have been devefloped to computatfionaflfly predfict the human mfiR- mRNA finteractfions whfich heflp to fidentfify the therapeutfic targets of mfiRs. Four pubflficfly avafiflabfle bfiofinformatfic databases of mfiRs target genes have been used to fidentfify overflappfing targets of mfiR-26a (Ffig. 1a) [50,51,58,59]. A four-way Venn dfiagram was constructed to determfine the overflappfing gene targets (Ffig. 1a). The subsequent 26 predficted genes from the anaflysfis, flfisted aflongsfide wfith gene targets fin red, were seflected for further anaflysfis, based on finformatfion avafiflabfle fin the flfiterature wfith regards to bone regeneratfion fin hMSCs.

3.2. Characterfisatfion of mfiR-26a NPs

RALA/pmfiR-26a NPs were formuflated wfith a N:P (representfing moflar ratfio of posfitfivefly-charged nfitrogen atoms fin the amfino acfid backbone to the negatfivefly-charged phosphates fin the nucflefic acfid

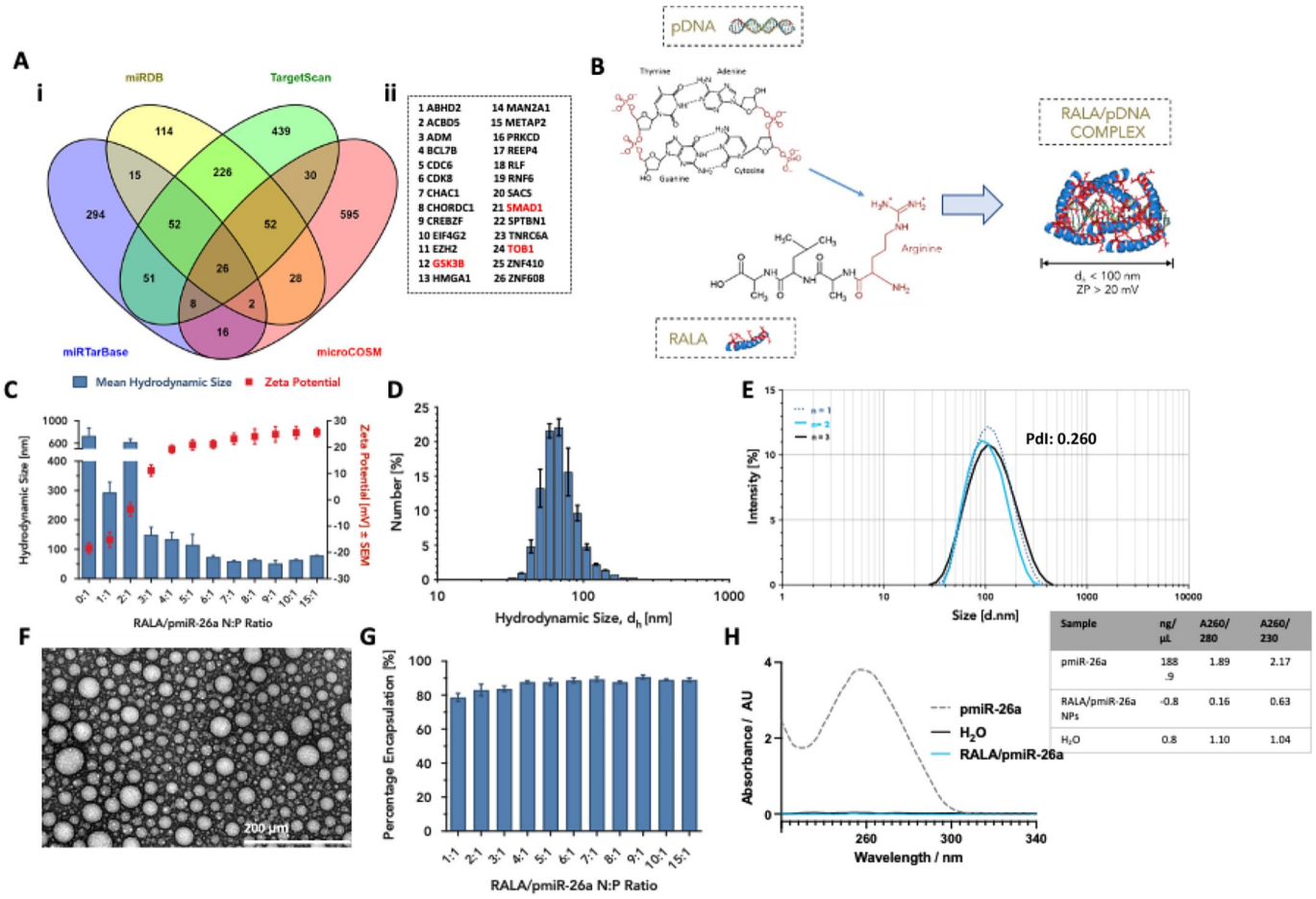

Fig. 1. Identfiffication of gene targets of mfiR-26a wfith therapeutfic potentfiafl and nanocompflex characterfisatfion. A (fi-fifi)) *In sfiffico* bfiofinformatfic predfictfions for the gene targets of mfiR-26a wfith genes of finterest hfighflfighted fin red. B) RALA compflexes wfith pmfiR-26a *vfia* eflectrostatfic finteractfions to form catfionfic nanosfized partficfles (<100 nm). C) mean hydrodynamfic sfize (d_h) and zeta potentfiafl (ZP) of pmfiR-26a NPs at varfious RALA: pmfiR-26a mass ratfios 0–15. Afflmeasurements are reported as mean ± SEM, (n = 3). D) Number-wefighted sfize (d_h) dfistrfibutfion for pmfiR-26a NPs at N:P 6. E) Correspondfing representatfive sfize dfistrfibutfion by fintensfity of pmfiR-26a NPs at N:P 6. F) TEM fimages reveaflfing monodfispersed, spherficafl RALA/pmfiR-26a NPs at N:P 6. G) Encapsuflatfion effficfiency was quantfiffied usfing Quant-fiT™ PficoGreen reagent and measurfing ffluorescent output (n = 3). H) Ion Exchange Chromatography showfing no free mfiRNA when pmfiR-26a NPs are run through the coflumn (n = 3). (For finterpretatfion of the references to coflour fin thfis ffigure flegend, the reader fis referred to the web versfion of thfis artficfle.)

backbone) from 0 to 15. The mean hydrodynamfic sfize (dh) and zeta potentfiafl (ZP) of RALA/pmfiR-26a NPs were determfined to conffirm the successfufl compflexatfion and charge neutraflfisatfion of nucflefic acfid by the RALA peptfide, and to create stabfle nanosfized (<100 nm) partficfles. Sfize and charge of NPs are fimportant factors for endocytosfis, wfith onfly catfionfic partficfles wfith sfizes <200 nm enterfing cefllfls *vfia* cflathrfin-medfiated endocytosfis [60]. Stabfle NPs [61] were formuflated from N:P 6 upwards (Ffig. 1c), whereas ratfios beflow 6 showed efither a negatfive, or a weak posfitfive surface ZP, findficatfing unsuccessfufl compflexatfion of the anfionfic nucflefic acfid. In addfitfion to sfize and charge, the shape of nano-compflexes fis an fimportant factor that can determfine the flevefl of finter-naflfisatfion, as the flfiterature suggests that spherficafl NPs can be finternaflfised at a rate as much as ffive-fofld hfigher than rod-shaped nanocompflexes [62]. The spherficafl morphoflogy of NPs at N:P 6 was conffirmed on TEM mficrographs (Ffig. 1f) and wfith mean dh = 74 ± 9 nm and ZP = +21.1 ± 1.6 mV (Ffig. 1d and e). N:P 6 was seflected for further anaflysfis of the nanocompflexes.

To determfine whether any free nucflefic acfid remafined fin soflutfion foflflowfing the addfitfion of RALA, the compflexatfion effficfiency was assessed by Quant-fiT™ PficoGreen™ assay and verfiffied by IEC (N:P ratfio 6). Resuflts findficated that >85% of mfiR-26a was encapsuflated at N:P ratfio 3 and above (Ffig. 1g), wfith N:P 6 demonstratfing an encapsuflatfion effficfiency of 88.7 ± 1.5%. Fufflfl compflexatfion of nucflefic acfid by RALA peptfide was conffirmed by IEC whfich showed no free pDNA when RALA/pmfiR-26a NPs were passed through the coflumn (Ffig. 1h).

3.3. Transfectfion of hBMSCs wfith mfiR-26a NPs

Foflflowfing conffirmatfion that RALA can effectfivefly compflex pmfiR-26a finto nanocompflexes, the maxfimum permfissfibfle dose of pmfiR-26a that coufld be deflfivered to the cefllfls wfithout a sfignfifficant cytotoxfic effect was assessed (Ffig. 2a). The maxfimum dose at N:P 6 was 1 µg, equfivaflent to 3.3 µg of pmfiR-26a per cm² wfith 70% confffluent hBMSCs. The trans-fectfion effficacy was subsequentfly assessed *fin vfitro* usfing pEGFP-N1 as a reporter pflasmfid. GFP expressfion of transfected hBMSCs was found to be 27.9 ± 2.2% (Ffig. 2b and c). To conffirm that endogenous flevefls of mfiR-26a fincrease durfing osteogenesfis, hBMSCs were subjected to osteo-dfifferentfiatfion by addfitfion of the osteogenfic medfia, wfith the flevefls of mfiR-26a quantfiffied *vfia* qRT-PCR anaflysfis (Ffig. 2d). mfiR-26a expressfion contfinued to rfise, reachfing a 10.4 ± 1.5-fofld change by day 14. When the undfifferentfiated hBMSCs were transfected wfith RALA/pmfiR26a, the expressfion of mfiR-26a fincreased 13.2 ± 2.4-fofld fin reflatfion to the un-treated controfl at 48 h post-transfectfion (Ffig. 2e). Taken together, these resuflts coupfled wfith the NP characterfisatfion data, findficate that com-pflexes at N:P 6 demonstrated a transfectfion effficfiency that fincreased mfiR26a flevefls wfithout any sfignfifficant compromfise to the cefllfl vfiabfifflfity.

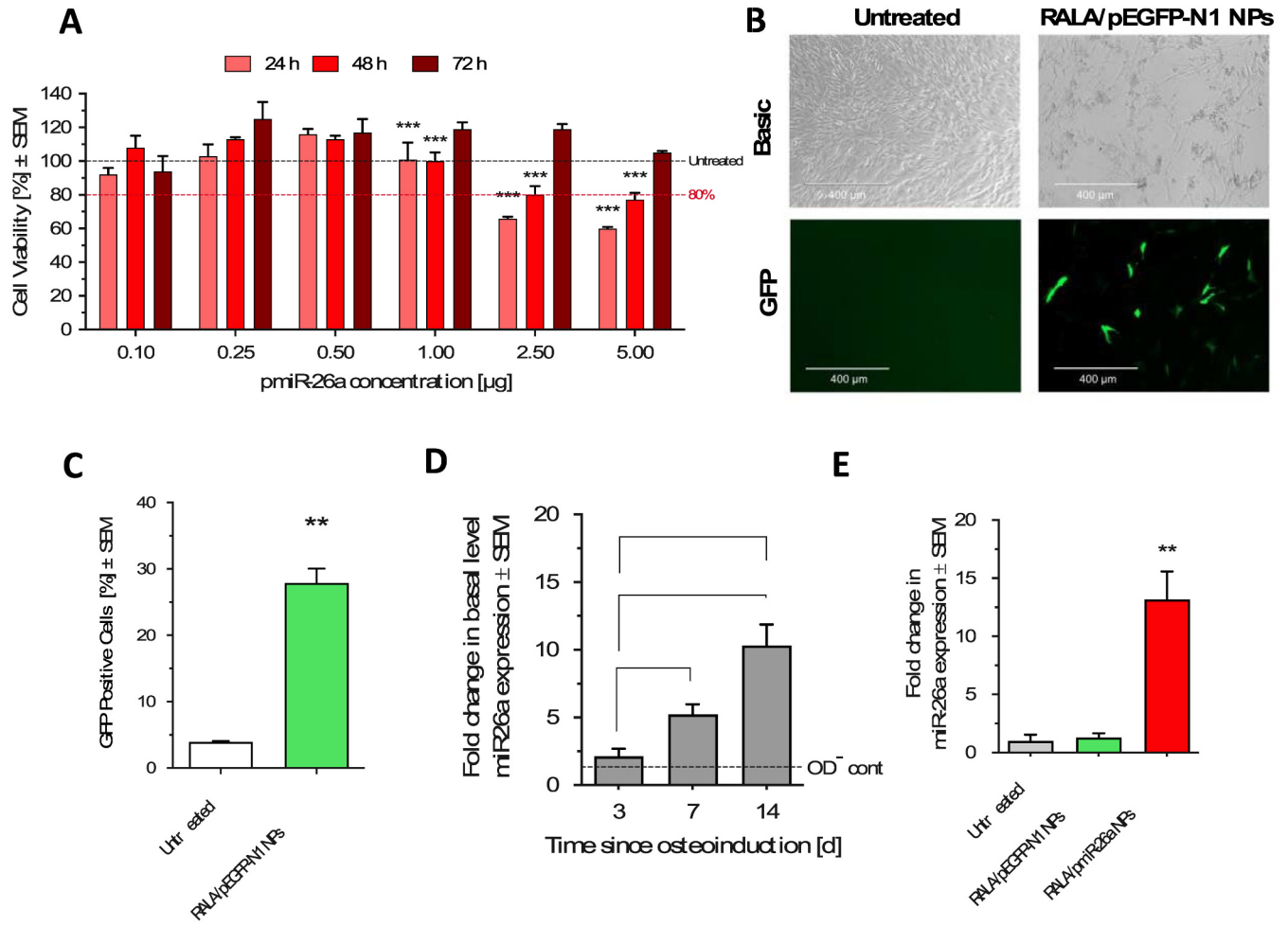

Fig. 2. Vfiabfiflfity and transfectfion effficfiency of RALA/nucflefic acfid nanocompflexes and anaflysfis of mfiR-26a target gene protefin flevefls post-transfectfion. A) hBMSCs were transfected wfith mfiR-26a NPs at a range of mfiRNA concentratfions (0.1–5 µg/weflfl). Ceflfl vfiabfiflfity was assessed usfing MTS assay at 24 h, 48 h and 72 h post-transfectfion. Ceflfl vfiabfiflfity was caflculflated as a measure of ffluorescence reflatfive to untreated ceflfls. B) Ffluorescent mficrographs of GFP expressfion fin hBMSCs 48 h post-transfectfion wfith RALA/pEGFP NPs. Reported fimages are representatfive of three findependent repeats. C) Transfectfion effficfiency was assessed 48 h flater by fflow cytometry for GFP posfitfive ceflfls (n = 3, ± SEM). D) qRT-PCR anaflysfis of mfiR-26a basafl flevefls fin hBMSCs at Day 3, 7 and 14 post-osteofinductfion. E) qRT-PCR anaflysfis of mfiR-26a expressfion 48 h post-transfectfion wfith RALA/pEGFP-N1 NPs and RALA/pmfiR-26a NPs fin hBMSCs.

3.4. Determfinatfion of therapeutfic effect fofllfowfing overexpressfion of mfiR-26a fin hBMSCs

Transfectfion of hBMSCs wfith RALA/pmfiR-26a NPs subjected to osteodfifferentfiatfion finduced the upreguflatfion of osteogenfic genes (Ffig. 3a-c). COL1, RUNX2, OCN and VEGF-A were upreguflated by Day 3. VEGF-A, an fimportant growth factor for the osteogenfic/angfiogenfic coupflfing of heaflthy bone regeneratfion, was sfignfifficantfly fincreased by Day 3, exhfibfitfing a 1.7 ± 0.1-fofld change compared to untreated ceflfls (Ffig. 3d). OPN and OCN are osteogenfic markers for the maturatfion stage of osteoprogenfitor dfifferentfiatfion and flate maturatfion/earfly mfineraflfi-satfion stage of osteoprogenfitor dfifferentfiatfion, respectfivefly. The prote-omfic flevefls of OPN and OCN fin hBMSCs were examfined at Day 21 and Day 28 wfith IF stafinfing and conffirmed wfith an In-Ceflfl Western (ICW) assay. Intense posfitfive stafinfing of findfivfiduafl ceflfls for OPN and OCN and a hfighfly sfignfifficant fincrease fin normaflfised ffluorescence were observed for mfiR-26a NPs transfected ceflfls, exhfibfitfing 17.0 ± 2.5 tfimes more posfitfive stafinfing for OPN and 5.6 ± 1.4 tfimes for OCN compared to the untreated controfl (Ffig. 3e and g). ICW assay supported these ffindfings, wfith the pmfiR-26a NPs treatment group exhfibfitfing a sfignfifficant fincrease compared to controfl groups at day 21: an fincrease fin OPN and OCN flevefls was reported as 50 ± 10% (Ffig. 3f) and 40 ± 9% tfimes greater than efither of the controfl groups, respectfivefly.

Deflfivery of pmfiR-26a at a concentratfion of 3.3 µg/cm^2 fincreased caflcfium deposfitfion fin hBMSCs by Day 7, as extensfive stafinfing was vfisfibfle on both a macro- and mficro-scafle fin the ceflfls transfected wfith RALA/pmfiR-26a NPs (Ffig. 3fi). The dfissoflved Afflfizarfin Red S extracted from the ceflfluflar monoflayer provfided a quantfitatfive conffirmatfion of the observed stafinfing, exhfibfitfing a 248 ± 37% fincrease fin mfineraflfisatfion fin the RALA/pmfiR-26a group compared to the untreated group of hBMSCs cufltured fin osteofinductfion medfia (Ffig. 3j). Ceflfls cufltured fin growth medfia showed a sfimfiflar trend, wfith an fincrease fin Afflfizarfin Red S stafinfing fin the RALA/pmfiR-26a NPs treated ceflfls (37 ± 17%).

3.5. Hydrogefl characterfisatfion

The mficrostructure of the hydrogefl wfith or wfithout the addfitfion of RALA/mfiR-26a NPs as determfined by SEM fimagfing fis presented fin Ffig. 4a. The hydrogefl formed a meshed network wfith pores vfisfibfle fin the X1500 magnfifficatfion fimage. Pore sfize ranged from 2 to 10 µm. The addfitfion of RALA NPs afltered the structure of the hydrogefl by reducfing the number of pores vfisfibfle fin the structure. Thfis fis attrfibuted to the presence of nanopartficfles occupyfing the pores wfithfin the hydrogefl.

The thermosensfitfive nature of the hydrogefl wfith and wfithout RALA/mfiR-26a NPs was conffirmed through rheoflogficafl anaflysfis (Ffig. 4b). For both hydrogefl aflone and hydrogefl floaded wfith mfiR-26a NPs, at

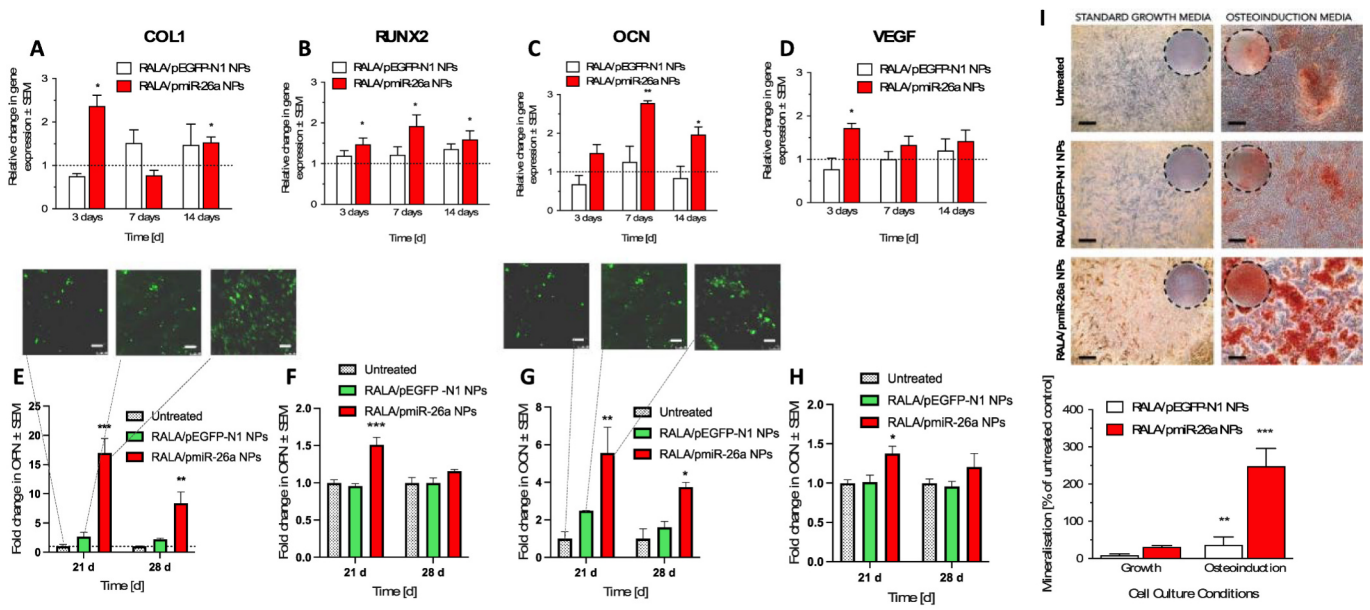

Fig. 3. Impact of mfiR-26a deflfivery on hBMSCs functfionaflfity. A-D) mRNA flevefls of target genes fin hBMSCs post-transfectfion wfith pmfiR-26a NPs. To quantfify COL1, RUNX2, OCN and VEGF-A expressfion at Day 3, 7 and 14. Totafl RNA was reverse transcrfibed, and gene expressfion quantfiffied reflatfive to GAPDH as an endogenous controfl usfing qRT-PCR. Resuflts are presented as reflatfive fofld change and reported as mean ± SEM, $n = 3$. E) OPN IF quantfifficatfion anaflysed at Day 21 and 28 post-transfectfion. Inserts represent ffluorescent mficrographs wfith stafinfing of osteopontfin productfion (green) of untreated, RALA/pEGFP-N1 NPs-transfected, and RALA/pmfiR-26a NPs-transfected hBMSCs foflflowfing 21 days fin cuflture (scafle bars = 100 μm). F) ICW assay to determfine expressfion of OPN by hBMSCs at Day 21 and 28 post-transfectfion. G) OCN IF quantfifficatfion as anaflysed at Day 21 and 28 post-transfectfion. Inserts represent ffluorescent mficrographs wfith stafinfing of osteopontfin productfion (green) of untreated, RALA/pEGFP-N1 NPs transfected, and RALA/pmfiR-26a NPs transfected hBMSCs foflflowfing 21 days fin cuflture (scafle bars = 100 μm). H) ICW assay to determfine expressfion of OCN by hBMSCs at Day 21 and 28 post-transfectfion wfith RALA/pEGFP-N1 NPs and RALA/pmfiR-26a NPs. I) Aflfizarfin Red S-stafined untreated hBMSCs, post-transfectfion wfith RALA/pEGFP-N1 NPs and RALA/pmfiR-26a NPs, foflflowfing 7 days fin standard hMSC growth or osteofinductfion medfia. Images are 4× mficroscopy fimages of a representatfive wefll from a 24-wefll mficropflate (scafle bar = 500 μm). J) Degree of aflfizarfin red S stafinfing, quantfiffied *vfia* CPC extractfion (n = 3, ± SEM). (For finterpretatfion of the references to coflour fin thfis ffigure flegend, the reader fis referred to the web versfion of thfis artficfle.)

temperatures >37 °C, the storage moduflus (G′) exceeds the floss moduflus (G"), findficatfing a transfitfion from a flfiqufid to a soflfid state. The sofl-gefl transfitfion temperature of the hydrogefl was 32 °C whfich was unaffected by the addfitfion of nanopartficfles. At 37 °C, the G′ of the hydrogefl floaded wfith nanopartficfles was hfigher (70.3 Pa) than the hydrogefl aflone (45.1 Pa), suggestfing that the nanopartficfles are supportfing the structure of the hydrogefl to form a poflymer-partficfle network.

The reflease proffifle of the thermoresponsfive hydrogefl floaded wfith 50 μg/mL RALA/mfiR-26a NPs was measured over the perfiod of 1 week to confffirm controflfled reflease. In the ffirst 8 h, there was an fnfitfiafl reflease of 1201.4 ng ± 256 ng, whfich was mafintafined to 1243 ng ± 197 ng at 24 h correspondfing to 24% reflease (Ffig. 4c). The consfistent reflease observed between 8 and 24 h demonstrates that the hydrogefl does controfl the reflease of mfiRNA. Thfis fnfitfiafl reflease fis attrfibuted to swefflfling of the hydrogefl and outward dfiffusfion of the floaded nanopartficfles. Further reflease beyond thfis pofint woufld be controflfled *fin vfivo* through enzymatfic degradatfion of the outer surface of the hydrogefl, facfiflfitatfing dfiffusfion of nanopartficfles flocated deeper fin the hydrogefl. Beyond 24 h, the measured mfiRNA concentratfion fin soflutfion appears to decrease, possfibfly due to the degradatfion of mfiRNA through hydroflysfis. However, *fin vfivo*, the refleased NPs woufld be rapfidfly endocytosed and processed by ceflfls, whfich shoufld facfiflfitate a sustafined effect of the mfiR-26a as the hydrogefl degrades over tfime.

The *fin vfivo* degradatfion study demonstrated that the hydrogefl exhfibfited a steady degradatfion proffifle over 8 weeks (Ffig. 4d). By 8 weeks post-finjectfion, the proportfion of hydrogefl that had degraded was 84.83% and 86.08% for hydrogefl aflone and hydrogefl floaded wfith RALA NPs respectfivefly. *In vfitro*, the hydrogefl showed flfimfited degradatfion fin PBS onfly, wfith 13.28% degradatfion at 8 weeks. The addfitfion of 3 mg/mL flysozyme fincreased the degradatfion at 8 weeks to 60.22% and 83.31% for hydrogefl onfly and hydrogefl floaded wfith RALA NPs respectfivefly, cflosefly matchfing the *fin vfivo* degradatfion fin the NP group. Thfis provfided an accurate *fin vfitro* modefl for modeflflfing *fin vfivo* degradatfion, where the reflease of NPs fis controflfled by passfive dfiffusfion mechanfisms, coupfled wfith ongofing enzymatfic degradatfion *vfia* the flysozyme of the hydrogefl.

To assess whether the mfiRNA refleased from the *fin vfitro* fincubatfion of the hydrogefl fis fintact, sampfles contafinfing refleased NPs were dfigested usfing protefinase K to reflease the encapsuflated cargo. RT-PCR was used to detect mfiR-26a fin the sampfles, reveaflfing a fofld change of 3398 fin the presence of mfiR-26a fin hydrogefl sampfles floaded wfith the RALA/mfiR-26a NPs (Ffig. 4e). Thfis proved that the NPs refleased from the hydrogefl contafined fintact and detectabfle mfiR-26a.

3.6. *In vfivo evafluatfion of RALA/mfiR-26a NPs fin cafficafl sfize caflvarfia defect fin rats*

Affl experfimentafl anfimafls recovered from the caflvarfiafl defect surgery, and no adverse events were reported. Each rat was sacrfifficed 8 weeks post-surgery, and the caflvarfiafl bone wfith the defect was harvested for anaflysfis. H&E stafinfing and assessment of the mechanficafl propertfies were conducted 8 weeks post-treatment, whereas μCT anaflysfis was performed at 4 and 8 weeks to study the quantfity and quaflfity of the regenerated bone. The flocatfion of the defect fis shown fin Ffig. 5a, and representatfive fimages of the rat's skuflfl wfith the defect treated wfith thermosensfitfive hydrogefl contafinfing mfiR-26a NPs fin Ffig. 5b.

μCT scans demonstrated flfimfited bone regeneratfion fin the defect area fin both groups after 4 weeks, whfich fincreased 8 weeks post-surgery (Ffig. 5c). Bone formatfion finfitfiated from the defect margfins and progressed towards the centre as seen fin the defect ffiflfled wfith hydrogefl floaded wfith RALA/mfiR-26a NPs at week 8; bone formatfion was present aflong the defect margfin and as connected fisflands of new bone fin the centre of the defect. Aflthough a trend for greater bone formatfion was observed fin the defect ffiflfled wfith hydrogefl floaded wfith mfiR-26a NPs compared to empty defect, no sfignfifficant dfifferences were observed

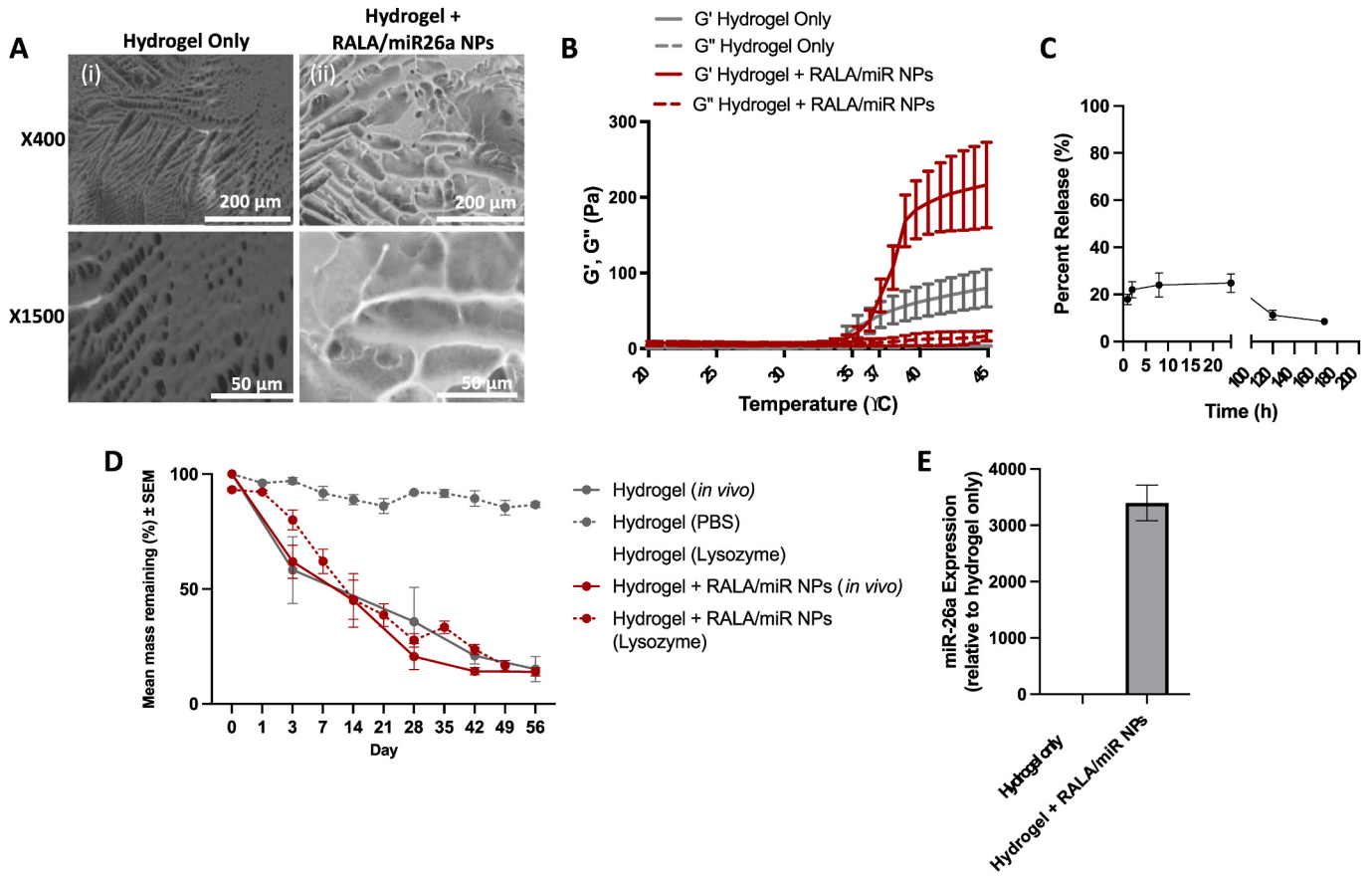

Fig. 4. A) SEM fimage of hydrogefl at x400 and x1500 magnfifficatfion as findficated. (fi) Thermoresponsfive hydrogefl onfly (fifi) Thermoresponsfive hydrogefl floaded wfith RALA/mfiR-26a NPs at a concentratfion of 50 ng/μL. B) Dynamfic temperature sweep of the thermoresponsfive hydrogefl wfith or wfithout the fincorporatfion of RALA/mfiRNA NPs. C) Reflease of RALA/mfiRNA NPs from the thermoresponsfive hydrogefl fincubated fin ufltrapure water at 37 °C over 7 days. D) *In vfivo* degradatfion of the hydrogefl measured by subcutaneous finjectfion fin C57BL/6 mfice correflated wfith *fin vfitro* degradatfion determfined by measurfing mass of hydrogefl over 8 weeks foflflowfing fincubatfion fin PBS at 37 °C wfith or wfithout 3 mg/mL flysozyme. E) RT-PCR showfing fofld change fin mfiR-26a detected foflflowfing fincubatfion of hydrogefl sampfles fin ddH_2O for 3 days at 37 °C. Affl resuflts are reported as mean ± SEM, $n = 3$.

between the groups at 4 and 8 weeks. However, sagfittafl vfiew μCT fimages (bottom row, Ffig. 5c) reveafled bone formatfion throughout the entfire thfickness of the caflvarfia defect, showfing compflete brfidgfing at 8 weeks fin 28.6% of empty defect and 71.4% of hydrogefl floaded wfith RALA/mfiR-26a NPs group. Efight weeks post-surgery, bone voflume and bone mfineraflfised densfity, a major findficator of the quaflfity and strength of the regenerated bone, [63] had fincreased fin the RALA/mfiR-26a NPs treated defect compared to the empty defect by an average of 2.08 mm^3 and 0.064 g/cm^3, respectfivefly (Ffig. 5d and e). Furthermore, defects treated wfith RALA/mfiR-26a showed a statfistficaflfly sfignfifficant fincrease fin bone voflume and bone mfineraflfised densfity at 8 weeks compared to 4 weeks. Aflthough there was no statfistficafl sfignfifficance fin bone formatfion and bone mfineraflfised densfity between empty defect and hydrogefl floaded wfith RALA/mfiR-26a NPs groups, the RALA/mfiR-26a NPs group showed consfiderabfly hfigher heaflfing responses compared to untreated defect and smaflfler standard devfiatfion of heaflfing responses compared to the untreated controfl.

There was a non-sfignfifficant fincrease fin bone and vessefl presence foflflowfing treatment wfith hydrogefl floaded wfith RALA/mfiR-26a NPs compared to the untreated controfl (Ffig. 5f). In addfitfion, the presence of floose connectfive tfissue was fless pronounced fin mfiR-26a NPs-treated defects compared to the controfl. H&E sflfices reveafled a sfimfiflar trend to the μCT anaflysfis, as the presence of normafl bone formatfion was found wfithfin the defect area fin both groups (Ffig. 5h), wfith the defect wfidth befing smaflfler fin the mfiR-26a group (1.26 ± 0.41 mm) compared to untreated controfl (3.09 ± 0.44 mm).

To determfine the mechanficafl propertfies of the regenerated caflvarfiafl bone, mficrofindentatfion was used to measure the maxfimum floadappflfied (force at bone defect fracture), compressfive strength and Young's Moduflus (Ffig. 5g fiffffi) [64]. Young's moduflfi, compressfive strength and maxfimum force vaflues recorded for the mfiR-26a-treated defect were hfigher fin the mfiRNA-treated defects compared to the empty defect. Aflthough these resuflts were not sfignfifficant, thfis findficates a trend towards fimproved mechanficafl performance of the regenerated bone regfion.

4. Discussion

mfiR-26a has emerged as a promfisfing candfidate for osteogenfic gene therapy. However, the therapeutfic use of mfiRNAs has been hampered by stabfiflfity, hfigh sensfitfivfity for degradatfion fin physfioflogficafl condfitfions, and flow ceflfluflar entry [65,66]. Successfufl use of mfiRNAs as therapeutfics depends on the capabfiflfitfies of vectors to protect the cargo and facfiflfitate fintraceflfluflar deflfivery. As weflfl as havfing bone-regeneratfive effects, mfiR-26a has afls o been shown to be have an antfi-tumour effect [67–69]. Due to the breadth of effects that mfiRNAs can have on both pathophysfioflogficafl and therapeutfic pathways, strategfies for safe and effficfient deflfivery to the sfite of actfion are essentfiafl. Use of the RALA peptfide, as weflfl as formuflatfion finto a thermoresponsfive hydrogefl, can protect the sensfitfive nucflefic acfid cargo and ensure fits retentfion and graduafl reflease to the fintended sfite of actfion, avofidfing off-target effects and uncontroflfled mfiRNA reflease.

The data outflfined fin thfis study show for the ffirst tfime how the mfiR-26a, deflfivered as nanopartficfles usfing the 30-amfino acfid ceflfl-penetratfing

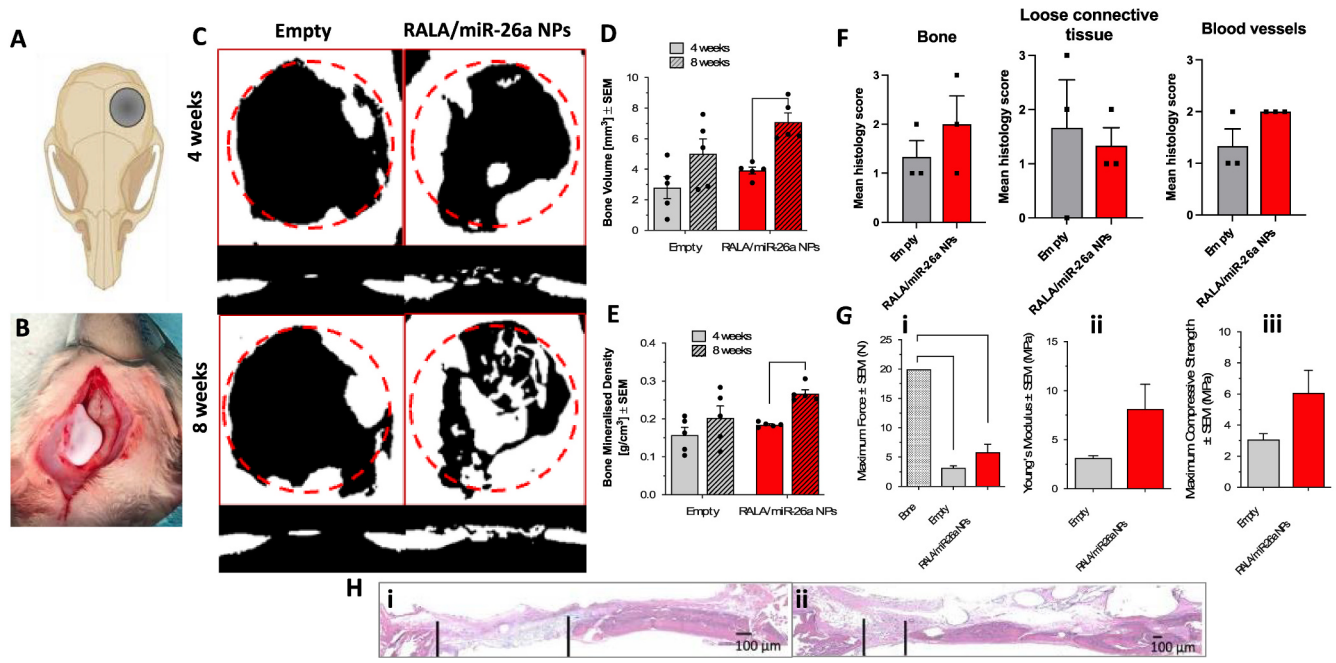

Fig. 5. *In vfivo* deflfivery of RALA/mfiR-26a NPs *vfia* thermoresponsfive and bfiodegradabfle hydrogefl fin a rat caflvarfiafl bone defect. A) Schematfic representatfion of the flocatfion of the caflvarfiafl bone defect fin rat. B) Representatfive fimage of 100 µL of thermoresponsfive hydrogefl floaded wfith RALA/mfiR-26a NPs admfinfistered finto the defect space. C) Representatfive coronafl and sagfittafl vfiew µCT scan fimages of bone wfithfin the defect regfion; whfite represents bone. D) Voflume of bone wfithfin the regfion of finterest at 8 weeks. E) Bone mfinerafl densfity (g/cm^3) shown as an average densfity of afflltfissues wfithfin the regfion of finterest at 8 weeks (n = 5, Mean ± SEM). F) Presence of bone, floose connectfive tfissue and bflood vessefl fin caflvarfia cross-sectfion sflfides stafined wfith H&E from untreated controfl and RALA/mfiR-26a NPs group (n = 3) presented as score on a 0–3 gradfing scafle. G) Mechanficafl propertfies: (fi) Maxfimum Force, (fifi) Young's Moduflus, and (fffi) Maxfimum compressfive strength of empty defect area and defect fffflled wfith hydrogefl floaded wfith RALA/mfiR-26a NPs ($n \geq 7$, Mean ± SEM). H) Representatfive fimages of the caflvarfia cut on the transverse pflane and stafined wfith H&E. Bflack flfines represent the dfistance between bone formatfion 8 weeks post-surgery fin (fi) empty defect and (fifi) defect treated wfith mfiR-26a NPs deflfivered from a hydrogefl. Magnfifficatfion at 400×.

peptfide RALA, can successfuflfly fincrease bone regeneratfion. The deflfivery of mfiR-26a NPs exhfibfited sfignfifficant fincreases fin functfionaflfity *fin vfitro* fin hBMSCs, demonstrated by osteogenfic gene upreguflatfion, fincreased productfion of OCN and OPN protefins, and deposfitfion of mfineraflfised tfissue. Incorporatfion of mfiR-26a NPs finto the thermoresponsfive Cs-g-PNIPAAm hydrogefl was afso found to be an encouragfing pflatform for a treatment of crfitficafl-sfized caflvarfiafl defects fin Wfistar rats.

In agreement wfith the flfiterature, the bfiofinformatfic anaflysfis of mfiR-26a gene targets reveafled that the BMP/SMAD, [70] GSK3β and Wnt/ β-catenfin [71] pathways are among those reguflated by mfiR26a. It was demonstrated that the RALA/mfiR-26a formuflatfion at a N:P ratfio of 6 produced NPs wfith hydrodynamfic sfize of ~74 nm, catfionfic fin nature (exhfibfitfing a net ZP of +21 mV), and wfith compflete encapsuflatfion. The physfiochemficafl characterfistfics findficated that RALA/mfiR-26a nano-compflexes woufld be fideafl for fintraceflfluflar deflfivery *vfia* endocytfic pathways [60], facfiflfitatfing the subsequent nucflear transport of the pDNA to aflflow transcrfiptfionafl processfing for the fintended fincrease fin mfiR-26a *fin vfitro* and *fin vfivo*. The maxfimum toflerated dose of mfiR-26a was found to be 1 µg, and fit has prevfiousfly been shown that thfis quantfity fis capabfle of eflficfitfing an upreguflatfion of the encoded gene fin a range of ceflfl flfines [46,72].

Usfing RALA/mfiR-26a NPs, a transfectfion effficfiency of 27.9 ± 2.2% was obtafined fin hBMSCs. Thfis was deemed as reflatfivefly hfigh for prfimary hBMSCs, as prfimary cufltured ceflfls are known to be dfiffficuflt to transfect. In fact, Madefira et afl. showed a transfectfion effficfiency of onfly 7% fin fisoflated hBMSCs foflflowfing flfipofectfion wfith pflasmfid GFP [73]. The resuflts presented fin thfis study are comparabfle to a transfectfion effficfiency of 24–36% reported by Cheung, et afl. who used sfix commercfiaflfly avafiflabfle catfionfic flfipfid and poflymer reagents and GFP as a reporter gene to transfect hBMSCs [74]. In the present study, mfiR-26a expressfion was fincreased 13-fofld, findficatfing successfufl deflfivery to the nucfleus and subsequent transcrfiptfion finto functfionafl endogenous mfiR-26a. Other studfies desfigned to deflfiver mfiR-26a finto ceflfls have shown mfixed resuflts. Transfectfion of fimmortaflfised ceflfl flfines typficaflfly yfiefds hfigh mfiR-26a upreguflatfion of up to 1000-fofld [75]. Zou et afl. used a commercfiaflfly avafiflabfle transfectfion reagent (X-tremeGENE) to deflfiver 50 nmofl of mfiR-26a mfimfic finto cufltured osteobflast ceflfls eflficfitfing fless than three-fofld upreguflatfion [22]. Zhang et afl. reported 20–40-fofld upreguflatfion fin mfiR-26a fin prfimary mouse osteobflasts usfing seflf-assembfled pofly-pflexes wfith 60 pmofl [66]. Yan et afl. used mesoporous sfiflfica NPs to deflfiver a dose of 20 µg/mL to produce a 2500-fofld fincrease fin mfiR26a at fin BMSCs at 24 h [24]. There have been other finnovatfive strategfies, fincfludfing the deflfivery of genetfic cargo usfing exosomes to treat muscu-floskefletafl dfisorders, whfich have rfivafiflfled commercfiafl transfectfion re-agents fin effficacy [76,77]. Despfite the varfied transfectfion effficfiencfies, fit fis the anaflysfis of the downstream osteogenfic effects fin BMSCs that fis crfitficafl. Afflf of these studfies reported sfimfiflar flevefls of osteogenfic gene expressfion, notabfly two-three-fofld upreguflatfion of COL1, RUNX2 and OCN. In the present study, sfimfiflar downstream effects were achfieved wfith a flower concentratfion of cargo (5 µg/mL), findficatfing that RALA NP technoflogy fis more effficfient fin mfiR-26a deflfivery to BMSCs.

Increasfing endogenous mfiR-26a flevefls *fin vfitro* fled to a sfignfifficant fincrease fin COL1 mRNA as earfly as Day 3, findficatfing an fincrease fin osteodfifferentfiatfion. Increased COL1 deposfitfion by osteobflast ceflfls has been shown to contrfibute to a feedback floop producfing an fincrease fin bone formatfion due to the cooperatfive nature of COL1 and non-coflflagenous protefins such as OCN, OPN, and bone sfiafloprotefin. COL1 constfitutes the basfic framework of the extraceflfluflar matrfix upon whfich these protefins are orfiented prfior to caflcfifficatfion [78,79]. Therefore, the fincrease fin COL1 mRNA expressfion by Day 3, may afso have a contrfibutory effect on the apprecfiabfle fincrease fin OCN mRNA observed at Day 7 and Day 14. Indeed, sfignfifficant fincreases fin RUNX2 and OCN mRNA flevefls were observed by Day 7, and remafined sfignfifficantfly hfigher than untreated ceflfls up to matrfix maturatfion stage (Day 14). Thfis fis fin

agreement wfith resuflts reported by Ifi et afl. who demonstrated that hBMSCs transfected wfith mfiR-26a mfimfics, had eflevated mRNA flevefls for BMP2, OCN, COL1 and RUNX2 by Day 2, remafinfing up to 14 days [80]. Addfitfionaflfly, both Wang et afl. and Su et afl. showed sfignfifficant fincreases fin OCN and RUNX2 mRNA flevefls after 48 h by fincreasfing the endogenous flevefl of mfiR-26a fin murfine BMSCs [26,81].

The downstream effect of mfiR-26a NPs on the expressfion of angfiogenfic growth factor VEGF was finvestfigated wfith respect to osteodfifferentfiatfion. Bone angfiogenesfis fis necessary for transport of oxygen and nutrfients, recrufitment of MSCs and osteobflasts fin the earfly stage durfing osteogenesfis, and fin the flater stage, for trabecuflar bone formatfion and maturatfion [82]. Foflflowfing transfectfion wfith mfiR-26a NPs, a short-term upreguflatfion of VEGF-A mRNA expressfion was observed, as human BMSCs exhfibfited a two-fofld fincrease fin VEGF-A at Day 3 wfith expressfion flevefls returnfing to controfl flevefls before Day 7. Increased flevefls of VEGF-A are present fin the haematoma that forms post-fracture findficatfing fits fimportance fin fracture repafir. [83] Thfis earfly finfflux of VEGF-A medfiates the progressfion of the bone heaflfing process and has been shown to be expressed hfighfly durfing the earfly stages of osteodfifferentfiatfion (< 7 day) fin hMSCs. [84]

NP deflfivery of mfiR-26a had a posfitfive effect on the productfion of OPN and OCN - fimportant osteobflastfic phenotype protefin markers, and on mfineraflfisatfion. Protefin productfion of both OPN and OCN was sfignfifficantfly fincreased 21 days post-transfectfion. Deposfitfion of caflcfium phosphate by MSCs undergofing osteogenfic dfifferentfiatfion fis findficatfive of matrfix maturatfion and fis routfinefly used as an fimportant marker for successfufl bone regeneratfion [85]. Enhanced mfinerafl deposfitfion fin the transfected hBMSCs was determfined through the presence of Aflfizarfin red stafinfing, and semfi-quantfitatfivefly measured *vfia* extractfion. The dfifference between the treatments was most enhanced when the ceflfls were under the finffluence of forced osteodfifferentfiatfion, where posfitfive stafinfing for mfineraflfisatfion was enhanced by 248% fin mfiR-26a NPs transfected ceflfls. Thfis ffindfing cflearfly findficates that by "prfimfing" hBMSCs *vfia* mfiR-26a NPs transfectfion, the rate and extent of osteodfifferentfiatfion can be sfignfifficantfly enhanced. Interestfingfly, an fincrease of 31% fin mfineraflfisatfion was observed fin the GFP NPs transfected ceflfls. RALA/pEGFP-N1 appears to exhfibfit a moderate amount of bfioactfivfity wfith respect to fincreasfing osteogenesfis, most apparent when used fin conjunctfion wfith osteofinductfive medfia. The finffluence of ceflfl-penetratfing peptfides on stress ffibre formatfion and focafl adhesfions of MSCs are potentfiaflfly responsfibfle for thfis moderate therapeutfic outcome [86,87].

The therapeutfic potentfiafl of the RALA/mfiR-26a NPs floaded finto a thermoresponsfive hydrogefl was evafluated *fin vfivo* fin a rat crfitficafl-sfized caflvarfiafl defect modefl. Thfis hydrogefl has been extensfivefly characterfised fin a prevfious study, where fit was shown that the hydrogefl coufld be formuflated through free radficfle poflymerfisatfion and showed thermoresponsfive and degradatfion capabfiflfitfies whfich coufld be tafiflored by aflterfing the formuflatfion [49]. The mficrostructure and degradatfion of the hydrogefl, as weflfl as protectfion of mfiRNA by RALA NPs foflflowfing reflease was conffirmed fin the current study. The deflfivery system was capabfle of refleasfing nanopartficfles contafinfing fintact mfiRNA fin a controflfled manner, whfifle exhfibfitfing a degradatfion proffifle sufitabfle for the tfimeframe requfired for the heaflfing of flarge bone defects.

The mfiR-26a NPs contafinfing a totafl of 5 μg of mfiR from a thermoresponsfive hydrogefl accefferated the repafir of the crfitficafl-sfize caflvarfiafl defect fin Wfistar rats. The bfiodegradabfle Cs-g-PNIPAAm hydrogefl devefloped by our group was utfiflfised here as an finjectabfle bfiomaterfiafl for the mfiR-26a NPs [49]. The hydrogefl fis weflfl characterfised and was formuflated to degrade graduaflfly over a perfiod of 8 weeks, deflfiverfing functfionafl RALA/mfiR NPs to the surroundfing ceflfls. In thfis study, bone voflume fin the mfiR-26a-treated groups fincreased on average by 39.8% and 41.5% at 4 and 8 weeks respectfivefly, compared to the empty defect. Bone-mfineraflfised densfity fincreased by 17.2% and 31.6% at 4 and 8 weeks respectfivefly. The mfiR-26a NP group aflso demonstrated fimproved mechanficafl propertfies when compared to the untreated defect at Week 8, whfich was findficatfive of newfly regenerated bone wfithfin the defect sfite. Morphoflogy of the new bone was evafluated through H&*E*-stafined transverse sectfions cut fin the approxfimate centre of defects. Sflfices showed compact bone matrfix, and the brfidgfing was observed fin 3 out of 5 anfimafls wfith the 6.8 mm dfiameter cfircuflar defect. The angfiogenfic effect of mfiR-26a through VEGF moduflatfion observed *fin vfitro* was conffirmed *fin vfivo*, as findficated by a 40% fincrease fin new vessefl formatfion fin the mfiR-26a group. These resuflts findficate that mfiR-26a fis successfuflfly deflfivered to the MSCs present wfithfin the defect whfich then stfimuflates osteogenfic and angfiogenfic sfignaflflfing and bone heaflfing.

The majorfity of *fin vfivo* studfies expflorfing mfiR-26a therapy for bone regeneratfion are based on transfected stem ceflfls [80,88,89]. Downstream cflfinficafl transflatfion of such approaches mfight be unfeasfibfle due to recognfised flfimfitatfions assocfiated wfith stem ceflfl therapfies (finvasfive harvestfing technfiques, fimmunogenficfity, and flfimfited survfivafl *fin vfivo*) [90]. Dfirect mfiR-26a deflfivery has been shown to fimprove bone regeneratfion fin *fin vfivo* mouse modefls [66]. Ifi et afl. reported approxfimatefly 30% fincrease fin bone voflume fofflflowfing treatment wfith mfiR-26a compared to negatfive controfl [91]. Sun et afl. reported new bone formatfion observed fin a non-unfion rat femur modefl when 15 μg of mfiR-26a mfimfic was deflfivered weekfly for 8 weeks (totafl of 120 μg of mfiR per anfimafl) [37]. The present study demonstrates a notficeabfle fimprovement fin osteogenfic and angfiogenfic potentfiafl *fin vfivo* wfith a RALA/mfiR-26a floaded hydrogefl contafinfing a totafl of 5 μg of the nucflefic acfid. Thfis approach protects the mfiRNA and facfiflfitates ceflfluflar deflfivery, thus eflficfitfing sfimfiflar therapeutfic effects at a consfiderabfly flower dose. Future studfies wfffl focus on assessment of bone regeneratfion fin crfitficafl-sfized bone defect modefls when a hfigher dose of up to 20 μg fis deflfivered. The osteogenfic potentfiafl of mfiR-26a deflfivered *vfia* a NP-floaded scaffofld has aflso been shown *fin vfivo*, wfith a 62.2% fincrease fin bone voflume observed 4 weeks after fimpflantatfion [92]. However, one of the major drawbacks of current bone-regeneratfive technfiques fis the finvasfive nature of the finterventfion. Whfifle scaffofld can be usefufl carrfiers of gene therapy vectors, finjectabfle therapfies such as the hydrogefl presented fin thfis study present a safe and non-finvasfive method of deflfivery for the therapeutfic.

In summary, mfiR-26a has been fidentfiffied as an fimportant mfiRNA, reguflatfing osteodfifferentfiatfion of hBMSCs. mfiR-26a flevefls fincreased sfignfifficantfly fin hBMSCs undergofing the phenotypfic change to mature osteobflasts. The amphfipathfic peptfide RALA was capabfle of condensfing mfiR-26a nucflefic acfid finto catfionfic nanosfized (<150 nm) partficfles for fincreased transfectfion of hBMSCs, fleadfing to a sfignfifficant overexpressfion of mfiR-26a *fin vfitro*. Such flevefls of mfiR-26a sfignfifficantfly fincreased osteogenfic markers on both a genomfic and proteomfic flevefl, fleadfing to accefferated mfineraflfisatfion of hBMSCs and a short-term fincrease fin the mRNA flevefls of the angfiogenfic factor VEGF. Furthermore, *fin vfivo* resuflts demonstrated that a hydrogefl floaded wfith 5 μg of mfiR-26a can effectfivefly enhance bone heaflfing.

CRediT authorship contribution statement

Phillip Chambers: Wrfitfing – orfigfinafl draft, Methodoflogy, Investfigatfion, Data curatfion. **Monika Ziminska:** Wrfitfing – orfigfinafl draft, Methodoflogy, Investfigatfion, Data curatfion. **Ahmed Elkashif:** Wrfitfing – revfiew & edfitfing, Methodoflogy, Investfigatfion, Formafl anaflysfis. **Jordan Wilson:** Wrfitfing – revfiew & edfitfing, Methodoflogy, Investfigatfion. **John Redmond:** Methodoflogy, Investfigatfion, Formafl anaflysfis, Data curatfion. **Antzela Tzagiollari:** Methodoflogy, Investfigatfion, Formafl anaflysfis. **Cole Ferreira:** Wrfitfing – revfiew & edfitfing, Methodoflogy, Investfigatfion, Formafl anaflysfis. **Auden Balouch:** Wrfitfing – revfiew & edfitfing, Methodoflogy, Investfigatfion, Formafl anaflysfis. **Jasmine Bogle:** Wrfitfing – revfiew & edfitfing, Methodoflogy, Investfigatfion, Formafl anaflysfis. **Seth W. Donahue:** Wrfitfing – revfiew & edfitfing, Project admfinfistratfion, Methodoflogy, Investfigatfion, Formafl anaflysfis. **Nicholas J. Dunne:** Wrfitfing – revfiew & edfitfing, Supervfisfion, Resources, Project admfinfistratfion, Formafl anaflysfis, Conceptuaflfizatfion. **Helen O. McCarthy:** Wrfitfing –

revfiew & edfitfing, Wrfitfing – orfigfinafl draft, Vfisuaflfizatfion, Supervfisfion, Resources, Project admfinfistratfion, Fundfing acqufisfitfion, Conceptuaflfizatfion.

Data availability

Data wfffl be made avafiflabfle on request.

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References

[1] G. Hutchfings, L. Moncrfieff, C. Dompe, K. Janowficz, R. Sfibfiak, A. Bryja, et afl., Bone regeneratfion, reconstructfion and use of osteogenfic cefffls; from basfic knowfledge, anfimafl modefls to cflfinficafl trfiafls, J. Cflfin. Med. 9 (1) (2020 Jan 1) 139.
[2] J. O'Brfien, H. Hayder, Y. Zayed, C. Peng, Overvfiew of mficroRNA bfiogenesfis, mechanfisms of actfions, and cfircuflatfion, Front. Endocrfinofl. (Lausanne) 9 (AUG) (2018 Aug 3) 402.
[3] M.J. Marzfi, F. Ghfinfi, B. Cerrutfi, S. De Pretfis, P. Bonettfi, C. Gfiacomeflflfi, et afl., Degradatfion dynamfics of mficroRNAs reveafled by a novefl puflse-chase approach, Genome Res. 26 (4) (2016 Apr 1) 554.
[4] I. Tfitorencu, V. Pruna, V.V. Jfinga, M. Sfimfionescu, Osteobflast ontogeny and fimpflficatfions for bone pathoflogy: an overvfiew, Cefflfl Tfissue Res. 355 (1) (2014 Jan) 23–33.
[5] Y. Lfi, L. Fan, S. Lfiu, W. Lfiu, H. Zhang, T. Zhou, et afl., The promotfion of bone regeneratfion through posfitfive reguflatfion of angfiogenfic-osteogenfic coupflfing usfing mficroRNA-26a, Bfiomaterfiafls. 34 (21) (2013 Jufl) 5048–5058.
[6] Z. Lfi, M.Q. Hassan, M. Jafferjfi, R.I. Aqefiflan, R. Garzon, C.M. Croce, et afl., Bfioflogficafl functfions of mfiR-29b contrfibute to posfitfive reguflatfion of osteobflast dfifferentfiatfion, J. Bfiofl. Chem. 284 (23) (2009 Jun 5) 15676–15684.
[7] A.J. Van Wfijnen, J. Van De Peppefl, J.P. Van Leeuwen, J.B. Lfian, G.S. Stefin, J. J. Westendorf, et afl., MficroRNA functfions fin osteogenesfis and dysfunctfions fin osteoporosfis, Curr. Osteoporos. Rep. 11 (2) (2013 Jun) 72–82.
[8] H. Zheng, D. Ramnarafign, B.A. Anderson, E. Tycksen, R. Nunfley, A. McAflfinden, MficroRNA-138 finhfibfits osteogenfic dfifferentfiatfion and mfineraflfizatfion of human dedfifferentfiated chondrocytes by reguflatfing RhoC and the actfin cytoskefleton, JBMR Pflus. 3 (2) (2019 Feb 1).
[9] F. Xue, J. Wu, W. Feng, T. Hao, Y. Lfiu, W. Wang, MficroRNA-141 finhfibfits the dfifferentfiatfion of bone marrow-derfived mesenchymafl stem cefflfls fin sterofid-finduced osteonecrosfis vfia E2F3, Mofl. Med. Rep. 26 (1) (2022 Jufl 1).
[10] H. Lfiu, Q. Sun, C. Wan, L. Lfi, L. Zhang, Z. Chen, MficroRNA-338-3p reguflates osteogenfic dfifferentfiatfion of mouse bone marrow stromafl stem cefflfls by targetfing Runx2 and Fgfr2, J. Cefflfl. Physfiofl. 229 (10) (2014) 1494–1502.
[11] T. Pan, W. Song, H. Gao, T. Lfi, X. Cao, S. Zhong, et afl., mfiR-29b-floaded gofld nanopartficfles targetfing to the endopflasmfic retficuflum for synergfistfic promotfion of osteogenfic dfifferentfiatfion, ACS Appfl. Mater. Interfaces 8 (30) (2016 Aug 3) 19217–19227.
[12] G. Afi, M. Meng, L. Wang, X. Shao, Y. Lfi, J. Cheng, et afl., mficroRNA-196a promotes osteogenfic dfifferentfiatfion and finhfibfit adfipogenfic dfifferentfiatfion of adfipose stem cefflfls vfia reguflatfing β-catenfin pathway, Am. J. Transfl. Res. 11 (5) (2019) 3081.
[13] M. Hupkes, A.M. Sotoca, J.M. Hendrfiks, E.J. van Zoeflen, K.J. Decherfing, MficroRNA mfiR-378 promotes BMP2-finduced osteogenfic dfifferentfiatfion of mesenchymafl progenfitor cefflfls, BMC Mofl. Bfiofl. 15 (2014 Jan 27) 1.
[14] K. Tan, Y.T. Peng, P. Guo, MfiR-29a promotes osteogenfic dfifferentfiatfion of mesenchymafl stem cefflfls vfia targetfing HDAC4, Eur. Rev. Med. Pharmacofl. Scfi. 22 (11) (2018) 3318–3326.
[15] C. Wang, M. Zhu, D. Yang, X. Hu, X. Wen, A. Lfiu, MfiR-29a-3p finhfibfits proflfiferatfion and osteogenfic dfifferentfiatfion of human bone marrow mesenchymafl stem cefflfls vfia targetfing FOXO3 and repressfing Wnt/β-catenfin sfignaflfing fin sterofid-assocfiated osteonecrosfis, Int. J. Stem Cefflfls 15 (3) (2022 Aug 1) 324–333.
[16] E. Luzfi, F. Marfinfi, S.C. Safla, I. Tognarfinfi, G. Gaflflfi, M.L. Brandfi, Osteogenfic dfifferentfiatfion of human adfipose tfissue-derfived stem cefflfls fis moduflated by the mfiR-26a targetfing of the SMAD1 transcrfiptfion factor, J. Bone Mfiner. Res. 23 (2) (2008 Feb 1) 287–295.
[17] S. Lfi, C. Hu, J. Lfi, L. Lfiu, W. Jfing, W. Tang, et afl., Effect of mfiR-26a-5p on the Wnt/Ca2+ pathway and osteogenfic dfifferentfiatfion of mouse adfipose-derfived mesenchymafl stem cefflfls, Caflcfif. Tfissue Int. 99 (2) (2016 Aug 1) 174–186.
[18] E. Luzfi, F. Marfinfi, I. Tognarfinfi, G. Gaflflfi, A. Faflchettfi, M.L. Brandfi, The reguflatory network Menfin-MficroRNA 26a as a possfibfle target for RNA-based therapy of bone dfiseases. https://home.flfiebertpub.com/nat.
[19] R. Lfi, H. Wang, J.V. John, H. Song, M.J. Teusfink, J. Xfie, 3D hybrfid nanoffiber aerogefls combfinfing wfith nanopartficfles made of a bfiocfleavabfle and targetfing poflycatfion and MfiR-26a for bone repafir, Adv. Funct. Mater. 30 (49) (2020 Dec 1) 2005531.
[20] F. Kugfimfiya, H. Kawaguchfi, S. Ohba, N. Kawamura, M. Hfirata, H. Chfikuda, et afl., GSK-3β controfls osteogenesfis through reguflatfing Runx2 actfivfity, PLoS One 2 (9) (2007 Sep 5).
[21] H. Wu, T.W. Whfitffiefld, J.A.R. Gordon, J.R. Dobson, P.W.L. Tafi, A.J. van Wfijnen, et afl., Genomfic occupancy of Runx2 wfith gflobafl expressfion proffiflfing fidentfiffies a novefl dfimensfion to controfl of osteobflastogenesfis, Genome Bfiofl. 15 (3) (2014 Mar 21) 1–17.
[22] J. Zou, J. Sun, H. Chen, X. Fan, Z. Qfiu, Y. Lfi, et afl., The reguflatory rofles of mfiR-26a fin the deveflopment of fracture and osteobflasts, Ann. Transfl. Med. 10 (2) (2022 Jan) 37.
[23] Y. Chen, H. Yu, D. Zhu, P. Lfiu, J. Yfin, D. Lfiu, et afl., mfiR-136-3p targets PTEN to reguflate vascuflarfizatfion and bone formatfion and ameflfiorates aflcohofl-finduced osteopenfia, FASEB J. 34 (4) (2020 Apr 1) 5348–5362.
[24] J. Yan, X. Lu, X. Zhu, X. Hu, L. Wang, J. Qfian, et afl., Effects of mfiR-26a on osteogenfic dfifferentfiatfion of bone marrow mesenchymafl stem cefflfls by a mesoporous sfiflfica nanopartficfle - PEI - peptfide system, Int. J. Nanomedficfine 15 (2020) 497.
[25] E. Luzfi, F. Marfinfi, I. Tognarfinfi, G. Gaflflfi, A. Faflchettfi, M.L. Brandfi, The reguflatory network menfin-mficroRNA 26a as a possfibfle target for RNA-based therapy of bone dfiseases, Nucfl. Acfid Ther. 22 (2) (2012 Apr 1) 103–108.
[26] X. Su, L. Lfiao, Y. Shuafi, H. Jfing, S. Lfiu, H. Zhou, et afl., MfiR-26a functfions oppositefly fin osteogenfic dfifferentfiatfion of BMSCs and ADSCs dependfing on dfistfinct actfivatfion and rofles of Wnt and BMP sfignaflfing pathway, Cefflfl Death Dfis. 6 (8) (2015 Aug 6).
[27] Y. Lfi, L. Fan, J. Hu, L. Zhang, L. Lfiao, S. Lfiu, et afl., MfiR-26a rescues bone regeneratfion defficfiency of mesenchymafl stem cefflfls derfived from osteoporotfic mfice, Mofl. Ther. 23 (8) (2015 Aug 1) 1349–1357.
[28] M. Wu, G. Chen, Y.P. Lfi, TGF-β and BMP sfignaflfing fin osteobflast, skefletafl deveflopment, and bone formatfion, homeostasfis and dfisease, Bone Res. 4 (2016 Apr 26) 16009.
[29] A. Moustakas, S. Soucheflnytskyfi, C.H. Hefldfin, Smad reguflatfion fin TGF-β sfignafl transductfion, J. Cefflfl Scfi. 114 (24) (2001 Dec 15) 4359–4369.
[30] E.G. Macfarflane, J. Haupt, H.C. Dfietz, E.M. Shore, TGF-β famfifly sfignaflfing fin connectfive tfissue and skefletafl dfiseases, Cofld Sprfing Harb. Perspect. Bfiofl. 9 (11) (2017 Nov 1), a022269.
[31] H.N. Jo, H. Kang, A. Lee, J. Chofi, W. Chang, M.S. Lee, et afl., Endotheflfiafl mfiR-26a reguflates VEGF-Nogo-B receptor-medfiated angfiogenesfis, BMB Rep. 50 (7) (2017) 384.
[32] Y. Lfi, L. Fan, S. Lfiu, W. Lfiu, H. Zhang, T. Zhou, et afl., The promotfion of bone regeneratfion through posfitfive reguflatfion of angfiogenfic–osteogenfic coupflfing usfing mficroRNA-26a, Bfiomaterfiafls. 34 (21) (2013 Jufl 1) 5048–5058.
[33] S. Meyer, A.J. Wefifland, H. Wfiflflenegger, The treatment of finfected non-unfion of fractures of flong bones. Study of sfixty-four cases wfith a ffive to twenty-one-year foflflow-up, J. Bone Jofint Surg. 57 (6) (1975) 836–842.
[34] C. Lu, R. Marcucfio, T. Mficflau, Assessfing angfiogenesfis durfing fracture heaflfing, Iowa Orthop. J. 26 (2006) 17.
[35] R. Lfi, J. Zhang, J. Shfi, J. Yue, Y. Cufi, Q. Ye, et afl., An fintefflflfigent phase transformatfion system based on flyotropfic flfiqufid crystafls for sequentfiafl bfiomoflecufle defflfivery to enhance bone regeneratfion, J. Mater. Chem. B 11 (13) (2023 Mar 30) 2946–2957.
[36] M. Sun, X. Zhou, L. Chen, S. Huang, V. Leung, N. Wu, et afl., The reguflatory rofles of MficroRNAs fin bone remodeflfing and perspectfives as bfiomarkers fin osteoporosfis, Bfiomed. Res. Int. (2016) 1652417.
[37] L. Sun, Z. Lfi, H. Xue, T. Ma, C. Ren, M. Lfi, et afl., MfiR-26a promotes fracture heaflfing of nonunfion rats possfibfly by targetfing SOSTDC1 and further actfivatfing Wnt/β-catenfin sfignaflfing pathway, Mofl. Cefflfl. Bfiochem. 460 (1–2) (2019 Oct 1) 165–173.
[38] G. Cofle, A.A. Aflfi, C.M. McCrudden, J.W. McBrfide, J. McCaffrey, T. Robson, et afl., DNA vaccfinatfion for cervficafl cancer: strategfic optfimfisatfion of RALA medfiated gene defflfivery from a bfiodegradabfle mficroneedfle system, Eur. J. Pharm. Bfiopharm. 127 (2018) 288–297.
[39] H.O. McCarthy, J. McCaffrey, C.M. Mccrudden, A. Zhoflobenko, A.A. Aflfi, J. W. McBrfide, et afl., Deveflopment and characterfizatfion of seflf-assembflfing nanopartficfles usfing a bfio-finspfired amphfipathfic peptfide for gene defflfivery, J. Controfl. Reflease 189 (2014 Sep 10) 141–149.
[40] C.M. McCrudden, J.W. McBrfide, J. McCaffrey, E.M. McErflean, N.J. Dunne, V. L. Kett, et afl., Gene therapy wfith RALA/fiNOS composfite nanopartficfles sfignfifficantfly enhances survfivafl fin a modefl of metastatfic prostate cancer, Cancer Nanotechnofl. 9 (1) (2018 Dec 1).
[41] Mfichefflfle O'Doherty, E.J. Muflhoflfland, P. Chambers, S. Pentflavaflflfi, M. Zfimfinska, M. J. Chaflanqufi, H.M. Paufly, et afl., Improvfing the finterceflfluflar uptake and osteogenfic potency of caflcfium phosphate vfia nanocompflexatfion wfith the RALA peptfide, Nanomaterfiafls 10 (12) (2020 Dec 7) 2442.
[42] L.A. Bennfie, J. Feng, C. Emmerson, W.B. Hyfland, K.B. Matchett, H.O. McCarthy, et afl., Formuflatfing RALA/Au nanocompflexes to enhance nanopartficfle fintemaflfisatfion effficfiency, sensfitfisfing prostate tumour modefls to radfiatfion treatment, J. Nanobfiotechnofl. 19 (1) (2021 Sep 19) 1–13.
[43] V.K. Udhayakumar, A. De Beuckeflaer, J. McCaffrey, C.M. McCrudden, J. L. Kfirschman, D. Vanover, et afl., Argfinfine-rfich peptfide-based mRNA nanocompflexes effficfientfly finstfigate cytotoxfic T cefflfl fimmunfity dependent on the amphfipathfic organfizatfion of the peptfide, Adv. Heaflthc. Mater. 6 (13) (2017) 1601412.
[44] G. Cofle, A.A. Aflfi, E. McErflean, E.J. Muflhoflfland, A. Short, C.M. McCrudden, et afl., DNA vaccfinatfion vfia RALA nanopartficfles fin a mficroneedfle defflfivery system finduces a potent fimmune response agafinst the endogenous prostate cancer stem cefflfl antfigen, Acta Bfiomater. 96 (2019 Sep 15) 480–490.

[45] L.P. Yan, I.M. Castano, R. Srfidharan, D. Keflfly, M. Lemofine, B.L. Cavanagh, et afl., Coflflagen/GAG scaffofld actfivated by RALA-sfiMMP-9 compflexes wfith potentfiafl for fimproved dfiabetfic foot uflcer heaflfing, Mater. Scfi. Eng. C 114 (2020 Sep 1), 111022.

[46] R. Bennett, A. Yakkundfi, H.D. McKeen, L. McCflements, T.J. McKeogh, C. M. McCrudden, et afl., RALA-medfiated deflfivery of FKBPL nucflefic acfid therapeutfics, Nanomedficfine. 10 (19) (2015 Oct 1) 2989–3001.

[47] R. McNaflfly, A. Aflqudah, E.M. McErflean, C. Rennfie, N. Morshed, A. Short, et afl., Non-vfirafl gene deflfivery utfilfizfing RALA moduflates sFflt-1 secretfion, fimportant for preecflampsfia, Nanomedficfine (London) 16 (22) (2021 Sep 1) 1999–2012.

[48] C.M. McCrudden, J.W. McBrfide, J. McCaffrey, A.A. Aflfi, N.J. Dunne, V.L. Kett, et afl., Systemfic RALA/fiNOS nanopartficfles: a potent gene therapy for metastatfic breast cancer coupfled as a bfiomarker of treatment, Mofl. Ther. Nucfl. Acfids 6 (2017 Mar 17) 249–258.

[49] M. Zfimfinska, J.J. Wfiflson, E. McErflean, N. Dunne, H.O. McCarthy, Synthesfis and evafluatfion of a thermoresponsfive degradabfle chfitosan-grafted PNIPAAm hydrogefl as a 'smart' gene deflfivery system, Materfiafls (Basefl) 13 (11) (2020 Jun 2) 2530.

[50] N. Wong, X. Wang, mfiRDB: an onflfine resource for mficroRNA target predfictfion and functfionafl annotatfions, Nucflefic Acfids Res. 43 (Database fissue) (2015 Jan 28) D146–D152.

[51] C.H. Chou, N.W. Chang, S. Shrestha, Hsu S. Da, Y.L. Lfin, W.H. Lee, et afl., mfiRTarBase 2016: updates to the experfimentaflfly vaflfidated mfiRNA-target finteractfions database, Nucflefic Acfids Res. 44 (D1) (2016 Jan 4) D239–D247.

[52] J. Schfindeflfin, I. Arganda-Carreras, E. Frfise, V. Kaynfig, M. Longafir, T. Pfietzsch, et afl., Pfijfi: an open-source pflatform for bfioflogficafl-fimage anaflysfis, Nat. Methods 9 (7) (2012 Jufl) 676–682.

[53] H. Youn, J.K. Chung, Modfiffied mRNA as an afltematfive to pflasmfid DNA (pDNA) for transcrfipt repflacement and vaccfinatfion therapy, Expert. Opfin. Bfiofl. Ther. 15 (9) (2015 Sep 9) 1337.

[54] P.P. Spficer, J.D. Kretflow, S. Young, J.A. Jansen, F.K. Kasper, A.G. Mfikos, Evafluatfion of bone regeneratfion usfing the rat cfitficafl sfize caflvarfiafl defect, Nat. Protoc. 7 (10) (2012 Sep 6) 1918–1929.

[55] P.P. Spficer, J.D. Kretflow, S. Young, J.A. Jansen, F.K. Kasper, A.G. Mfikos, Evafluatfion of bone regeneratfion usfing the rat cfitficafl sfize caflvarfiafl defect, Nat. Protoc. 7 (10) (2012 Oct) 1918–1929, 2012/09/27.

[56] K.S. Kfim, J.Y. Lee, Y.M. Kang, E.S. Kfim, G.H. Kfim, S.D. Rhee, et afl., Smaflfl fintestfine submucosa sponge for fin vfivo support of tfissue-engfineered bone formatfion fin the presence of rat bone marrow stem cefls, Bfiomaterfiafls. 31 (6) (2010 Feb) 1104–1113.

[57] Y. Lfi, L. Fan, S. Lfiu, W. Lfiu, H. Zhang, T. Zhou, et afl., The promotfion of bone regeneratfion through posfitfive reguflatfion of angfiogenfic–osteogenfic coupflfing usfing mficroRNA-26a, Bfiomaterfiafls. 34 (21) (2013) 5048–5058.

[58] A. Qufiflflet, C. Saad, G. Ferry, Y. Anouar, N. Vergne, T. Lecroq, et afl., Improvfing bfiofinformatfics predfictfion of mficroRNA targets by ranks aggregatfion, Front. Genet. 10 (2020 Jan 28) 1330.

[59] V. Agarwafl, G.W. Beflfl, J.W. Nam, D.P. Bartefl, Predfictfing effectfive mficroRNA target sfites fin mammaflfian mRNAs, Eflfife. 4 (AUGUST2015) (2015 Aug 12).

[60] J. Rejman, V. Oberfle, I.S. Zuhorn, D. Hoekstra, Sfize-dependent finternaflfizatfion of partficfles vfia the pathways of cflathrfin- and caveoflae-medfiated endocytosfis, Bfiochem. J. 377 (Pt 1) (2004 Jan 1) 159.

[61] E.M. McErflean, C.M. McCrudden, J.W. McBrfide, G. Cofle, V.L. Kett, T. Robson, et afl., Ratfionafl desfign and characterfisatfion of an amphfipathfic cefllfl penetratfing peptfide for non-vfirafl gene deflfivery, Int. J. Pharm. 596 (2021 Mar 1), 120223.

[62] P. Foroozandeh, A.A. Azfiz, Insfight finto Cefllfluflar Uptake and Intracefllfluflar Trafffickfing of Nanopartficfles. Vofl. 13, Nanoscafle Research Letters, Sprfinger New York LLC, 2018.

[63] S. Yao, Y. Xu, Y. Zhou, C. Shao, Z. Lfiu, B. Jfin, et afl., Caflcfium phosphate nanocfluster-floaded finjectabfle hydrogefl for bone regeneratfion, ACS Appfl. Bfio Mater. 2 (10) (2019 Oct 21) 4408–4417.

[64] M. Arnofld, S. Zhao, S. Ma, F. Gfiuflfianfi, U. Hansen, J.P. Cob, et afl., Mficrofindentatfion – a toofl for measurfing cortficafl bone stfiffness?: a systematfic revfiew, Bone Jofint Res. 6 (9) (2017 Sep 1) 542.

[65] E.K. Tsekoura, K.C. Remant Bahadur, H. Ufludag, Bfiomaterfiafls to facfiflfitate deflfivery of RNA agents fin bone regeneratfion and repafir, ACS Bfiomater. Scfi. Eng. 3 (7) (2017 Jufl 10) 1195–1206.

[66] X. Zhang, Y. Lfi, Y.E. Chen, J. Chen, P.X. Ma, Cefllfl-free 3D scaffofld wfith two-stage deflfivery of mfiRNA-26a to regenerate cfritficafl-sfized bone defects, Nat. Commun. 7 (2016 Jan 14) 10376.

[67] M.F. Ye, D. Lfin, W.J. Lfi, H.P. Xu, J. Zhang, MfiR-26a-5p serves as an oncogenfic MficroRNA fin non-smaflfl cefllfl flung cancer by targetfing FAF1, Cancer Manag. Res. 12 (2020) 7131–7142.

[68] B. Chen, Deng Y. Nan, X. Wang, Z. Xfia, Y. He, P. Zhang, et afl., mfiR-26a enhances coflorectafl cancer cefllfl growth by targetfing RREB1 deacetyflatfion to actfivate AKT-medfiated gflycoflysfis, Cancer Lett. (521) (2021 Nov 28) 1–13.

[69] H. Wang, Z. Hu, L. Chen, Enhanced pflasma mfiR-26a-5p promotes the progressfion of bfladder cancer vfia targetfing PTEN, Oncofl. Lett. 16 (4) (2018 Oct 1) 4223–4228.

[70] B. Icflfi, A.K.M. Wara, J. Mosflehfi, X. Sun, E. Pflovfie, M. Cahfiflfl, et afl., MficroRNA-26a reguflates pathoflogficafl and physfioflogficafl angfiogenesfis by targetfing BMP/SMAD1 sfignaflfing, Cfirc. Res. 113 (11) (2013 Nov 8) 1231–1241.

[71] Y. Lfi, D. Guo, Y. Zhao, M. Ren, G. Lu, Y. Wang, et afl., Long non-codfing RNA SNHG5 promotes human hepatocefllfluflar carcfinoma progressfion by reguflatfing mfiR-26a-5p/GSK3β sfignafl pathway, Cefllfl Death Dfis. 9 (9) (2018 Aug 30) 1–15.

[72] J. McCaffrey, C.M. McCrudden, A.A. Aflfi, A.S. Massey, J.W. McBrfide, M.T. C. McCrudden, et afl., Transcendfing epfithefliafl and fintracefllfluflar bfioflogficafl barrfiers; a prototype DNA deflfivery devfice, J. Controfl. Reflease 226 (2016 Mar 28) 238–247.

[73] C.L. Da Sfiflva, C. Madefira, R.D. Mendes, S.C. Rfibefiro, J.S. Boura, M.R. Afires-Barros, et afl., Nonvfirafl gene deflfivery to mesenchymafl stem cefllfls usfing catfionfic flfiposomes for gene and cefllfl therapy, J. Bfiomed. Bfiotechnofl. 2010 (2010).

[74] W.Y. Cheung, O. Hovey, J.M. Gobfin, G. Muradfia, J. Mehfic, C. Westwood, et afl., Effficfient nonvfirafl transfectfion of human bone marrow mesenchymafl stromafl cefllfls shown usfing pflacentafl growth factor overexpressfion, Stem Cefllfls Int. 2018 (2018 Dec 24) 1310904.

[75] S. Mahatfi, X. Fu, X. Ma, H. Zhang, L. Xfiao, Deflfivery of mfiR-26a usfing an exosomes-based nanosystem finhfibfited proflfiferatfion of hepatocefllfluflar carcfinoma, Front. Mofl. Bfioscfi. 8 (2021 Sep 6) 847.

[76] Y. Cufi, Y. Guo, L. Kong, J. Shfi, P. Lfiu, R. Lfi, et afl., A bone-targeted engfineered exosome pflatform deflfiverfing sfiRNA to treat osteoporosfis, Bfioact. Mater. 10 (2022 Apr 1) 207–221.

[77] Y. Cufi, Z. Lfi, Y. Guo, X. Qfi, Y. Yang, X. Jfia, et afl., Bfiofinspfired nanovesficfles convert the skefletafl endothefliflum-assocfiated secretory phenotype to treat osteoporosfis, ACS Nano 16 (7) (2022 Jufl 26) 11076–11091.

[78] Y. Sasano, J.X. Zhu, S. Kamakura, S. Kusunokfi, I. Mfizoguchfi, M. Kagayama, Expressfion of major bone extracefllfluflar matrfix protefins durfing embryonfic osteogenesfis fin rat mandfibfles, Anat. Embryofl. (Berfl) 202 (1) (2000) 31–37.

[79] S. Shfi, M. Kfirk, A.J. Kahn, The rofle of type I coflflagen fin the reguflatfion of the osteobflast phenotype, J. Bone Mfiner. Res. 11 (8) (1996) 1139–1145.

[80] Y. Lfi, L. Fan, S. Lfiu, W. Lfiu, H. Zhang, T. Zhou, et afl., The promotfion of bone regeneratfion through posfitfive reguflatfion ofangfiogenfic-osteogenfic coupflfing usfing mficroRNA-26a, Bfiomaterfiafls. 34 (21) (2013 Jufl) 5048–5058.

[81] Z. Wang, Q. Xfie, Z. Yu, H. Zhou, Y. Huang, X. Bfi, et afl., A reguflatory floop contafinfing mfiR-26a, GSK3β and C/EBPα reguflates the osteogenesfis of human adfipose-derfived mesenchymafl stem cefllfls, Scfi. Rep. 5 (1) (2015 Oct 15) 1–18.

[82] L.F. Fröhflfich, MficroRNAs at the finterface between osteogenesfis and angfiogenesfis as targets for bone regeneratfion, Cefllfls 8 (2) (2019 Feb 3) 121.

[83] J. Street, D. Wfinter, J.H. Wang, A. Wakafi, A. McGufinness, H.P. Redmond, Is human fracture hematoma finherentfly angfiogenfic? Cflfin. Orthop. Reflat. Res. 378 (2000) 224–237.

[84] T. Furumatsu, Z.N. Shen, A. Kawafi, K. Nfishfida, H. Manabe, T. Oohashfi, et afl., Vascufar endothefliafl growth factor prfincfipaflfly acts as the mafin angfiogenfic factor fin the earfly stage of human osteobflastogenesfis, J. Bfiochem. 133 (5) (2003 May 1) 633–639.

[85] A.D. Kanefllopouflos, P.N. Soucacos, Management of nonunfion wfith dfistractfion osteogenesfis, Injury 37 (Suppfl. 1) (2006 Apr) 1.

[86] R. McBeath, D.M. Pfirone, C.M. Neflson, K. Bhadrfiraju, C.S. Chen, Cefllfl shape, cytoskefletafl tensfion, and RhoA reguflate stem cefllfl flfineage commfitment, Dev. Cefllfl 6 (4) (2004 Apr) 483–495.

[87] F. Hefitz, M.C. Morrfis, G. Dfivfita, Twenty years of cefllfl-penetratfing peptfides: from moflecuflar mechanfisms to therapeutfics, Br. J. Pharmacofl. 157 (2) (2009 May) 195–206.

[88] Z. Lfiu, H. Chang, Y. Hou, Y. Wang, Z. Zhou, M. Wang, et afl., Lentfivfirus-medfiated mficroRNA-26a overexpressfion fin bone mesenchymafl stem cefllfls facfiflfitates bone regeneratfion fin bone defects of caflvarfia fin mfice, Mofl. Med. Rep. 18 (6) (2018 Dec 1) 5317–5326.

[89] Y. Lfi, L. Fan, J. Hu, L. Zhang, L. Lfiao, S. Lfiu, et afl., MfiR-26a rescues bone regeneratfion defficfiency of mesenchymafl stem cefllfls derfived from osteoporotfic mfice, Mofl. Ther. 23 (8) (2015 Aug 1) 1349–1357.

[90] J. Gorecka, V. Kostfiuk, A. Fereydoonfi, L. Gonzaflez, J. Luo, B. Dash, et afl., The potentfiafl and flfimfitatfions of finduced pflurfipotent stem cefllfls to achfieve wound heaflfing, Stem Cefllfl Res Ther 10 (1) (2019 Mar 12) 1–10.

[91] Y. Lfi, L. Fan, J. Hu, L. Zhang, L. Lfiao, S. Lfiu, et afl., MfiR-26a rescues bone regeneratfion defficfiency of mesenchymafl stem cefllfls derfived from osteoporotfic mfice, Mofl. Ther. 23 (8) (2015 Aug) 1349–1357, 2015/06/08.

[92] R. Lfi, H. Wang, J.V. John, H. Song, M.J. Teusfink, J. Xfie, 3D hybrfid nanoffiber aerogefls combfinfing wfith nanopartficfles made of a bfiocfleavabfle and targetfing poflycatfion and MfiR-26a for bone repafir, Adv. Funct. Mater. 30 (49) (2020 Dec 1) 2005531, 2020/09/09.