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Controlled and sustained delivery of siRNA/NPs from hydrogels expedites bone fracture healing

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Abstract

Despite great potential, delivery remains as the most significant barrier to the widespread use of siRNA therapeutics. siRNA has delivery limitations due to susceptibility to RNase degradation, low cellular uptake, and poor tissue-specific localization. Here, we report the development of a hybrid nanoparticle (NP)/hydrogel system that overcomes these challenges. Hydrogels provide localized and sustained delivery via controlled release of entrapped siRNA/NP complexes while NPs protect and enable efficient cytosolic accumulation of siRNA. To demonstrate therapeutic efficacy, regenerative siRNA against WW domain-containing E3 ubiquitin protein ligase 1 (*Wwp1*) complexed with NP were entrapped within poly(ethylene glycol) (PEG)-based hydrogels and implanted at sites of murine mid-diaphyseal femur fractures. Results showed localization of hydrogels and controlled release of siRNA/NPs at fractures for 28 days, a timeframe over which fracture healing occurs. siRNA/NP sustained delivery from hydrogels resulted in significant *Wwp1* silencing at fracture callus compared to untreated controls. Fractures treated with siRNA/NP hydrogels exhibited accelerated bone formation and significantly increased biomechanical strength. This NP/hydrogel siRNA delivery system has outstanding therapeutic promise to augment fracture healing. Owing to the structural similarities of siRNA, the development of the hydrogel platform for *in vivo* siRNA delivery has myriad therapeutic possibilities in orthopaedics and beyond.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.biomaterials.2017.06.001>

Keywords

Hydrogels; Nanoparticles; siRNA; Drug delivery; Fracture healing

1. Introduction

Small interfering RNA (siRNA) exhibits significant therapeutic potential as it can induce potent and specific silencing of a broad range of genetic targets [1–3]. However, clinical translation of siRNA-based therapies has been hampered by delivery barriers associated with susceptibility to nuclease degradation, cell and endosomal membrane impermeability at the target site, and lysosomal degradation [3,4]. While many carriers have been developed to overcome these challenges [5–7], the most prominent barrier is tissue/cell-specific delivery of siRNA. Systemic delivery of siRNA, which is typically achieved using nanoparticle (NP)-based delivery systems, suffers from inefficiencies due to liver accumulation and reticuloendothelial system (RES) clearance, resulting in subtherapeutic concentrations at target tissues [8]. The concomitant high doses of administered siRNA required to reach therapeutic levels result in off-target effects, limiting the safety of siRNA therapeutics [9].

The incidence and burden of fractures, of which approximately 20% have impaired healing [10], is predicted to increase dramatically as the population ages, motivating the development of potent therapeutic strategies to enhance fracture healing. Accordingly, targeting genes that negatively regulate bone formation without altering bone resorption or affecting other cell types may overcome impaired bone healing [11]. Therefore, siRNA has been employed to augment osteogenic function of mesenchymal stem cells (MSCs), which play a key role in fracture repair. For example, delivery of siRNA against dickkopf-related protein 1 (DKK1) [12] or guanine nucleotide binding protein alpha stimulating activity polypeptide 1 (GNAS1) [13] enhances osteogenic differentiation of MSCs. These data suggest that the delivery of siRNA can be used to enhance bone regeneration, through promotion of MSC differentiation, survival, and/or tissue production [14].

Hydrogels, which are three dimensional (3D) networks formed from crosslinked hydrophilic polymers, have great potential to sustain the delivery of biomolecules, including siRNA [15]. Hydrogels have been used for drug delivery to the heart, eyes, lungs, central nervous system, and bone [15–18]. For siRNA delivery specifically, dextran and chitosan-based hydrogels have been utilized [19,20]. While these approaches sustain siRNA release for up to 10–14 days, regenerative medicine approaches, especially for bone healing, commonly require gene knockdown for a month or longer. Subsequently, biomaterial delivery depots have been developed for siRNA to promote angiogenesis [21] and inhibit tumor growth [22]. Although these approaches proved feasibility of local siRNA delivery, the challenge of delivering therapeutic siRNA to the right tissue over times commensurate with healing (approximately 3–4 weeks) still remains. Recently, nanofiber polymer scaffolds were developed that achieve sustained micro-RNA (miRNA) delivery for 2–4 weeks [23]. The scaffolds improved calvarial bone defect regeneration, paving the way for the development of functional materials for delivery of nucleic acid-based drugs to bone.

Herein, we report that a hybrid nanoparticle (NP)/PEG hydrogel delivery system can be used to control the release of a regenerative siRNA targeting WW domain-containing E3 ubiquitin protein ligase 1 (*Wwp1*) (Fig. 1A – E) for fracture healing in a clinically-relevant fracture model. *Wwp1* has been previously identified as a negative regulator of bone formation, as increased bone formation rate and bone mass were observed in mice globally deficient in *Wwp1* [24]. *Wwp1* siRNA electrostatically interacts with polymer diblock nanoparticles (NP) (Fig. 1A) to form siRNA/NP complexes (Fig. 1B). siRNA/NPs were then embedded in hydrolytically degradable hydrogels made from PEG-*b*-poly(lactide)-*b*-dimethacrylate (PEG-*b*-PLA-*b*-DM) (Fig. 1C), which was designed to deliver siRNA/NPs to fractures in a sustained and localized manner (Fig. 1D). We showed that hydrogel-mediated siRNA delivery resulted in prolonged knockdown compared to siRNA/NP alone (Fig. 2). We also demonstrated sustained localization and release of siRNA/NP and hydrogels *in vivo* in a murine femoral fracture model (Fig. 3). Knockdown of *Wwp1* using siRNA/NPs hydrogels showed significantly increased bone formation and accelerated healing (Figs. 4–6), highlighting the therapeutic promise of this approach.

2. Materials and methods

2.1. Diblock copolymer synthesis and characterization

The siRNA delivery copolymer consisted of two blocks. The first cationic block was composed of dimethylaminoethyl methacrylate (DMAEMA), which is designed to protect and complex with negatively charged siRNA [25–27]. pDMAEMA was synthesized by reversible addition-fragmentation chain transfer (RAFT) polymerization as previously described using the radical initiator 2,20-Azobis(2-methylpropionitrile) (AIBN, Sigma) and chain transfer agent (CTA), ECT (4-cyano-4-[(ethylsulfanylthiocarbonyl)sulfanyl] pentanoic acid), under a nitrogen atmosphere in N,N-dimethylformamide (DMF) at 60 °C for 6 h. The second block, a tercopolymer of DMAEMA, propylacrylic acid (PAA), and butyl methacrylate (BMA), has been shown to be essential for endosomal escape of siRNA [25]. AIBN and pDMAEMA macroCTA were added to DMAEMA, PAA, and BMA (DMAEMA:PAA:BMA = 25:25:50) in DMF and the reaction was allowed to proceed at 60 °C for 24 h. The reaction was terminated via exposure atmospheric oxygen and the diblock polymers were purified via precipitation in 80:20 pentane:diethyl ether and dried under vacuum. First block and diblock molecular weights (Mn) was determined using gel permeation chromatography (GPC) (Shimadzu Technologies) using a TSKgel Guard SuperH-H guard column (Tosoh Biosciences) and a TSKgel Super HM-N for separation using a column oven at 60 °C. Polymer composition was determined using proton nuclear magnetic resonance (¹H-NMR, Bruker Avance400), as previously described [25].

2.2. siRNA/NP complexation and characterization

Diblock polymers were dissolved in ethanol at 4 mg/ml concentration then diluted to 2 mg/ml in PBS. The polymer solution was dialyzed in 3500 Da molecular cutoff dialysis tubing against distilled H₂O for 24 h. The concentration of the resulting polymer solution was then determined gravimetrically after lyophilization. NP size and zeta potential were characterized via dynamic light scattering analysis (DLS) using a Malvern Zetasizer. Results of NP characterization can be found in Table 1. The NP solution was filtered (0.2 μm) then

utilized to form complexes with Silencer[®] Select Negative Control No. 1 siRNA (Cat. # 4,390,843) and Silencer[®] Select *Wwp1* mouse siRNA s98891 (Cat. # 4,390,771) from Ambion[®], as previously described [25,26]. Briefly, siRNA, PBS, and then diblock copolymer were added to 1.5 mL Eppendorf tubes and incubated at room temperature for 20 min for complexation. The critical charge ratio indicating the ratio between the positive charges within the pDMAEMA block (0.5 positive charge per mole since 50% of the DMAEMA residues are protonated at physiological pH) and negative charges of siRNA (42 negative charges per mole) at which there is no uncomplexed siRNA was determined by gel electrophoresis [27].

2.3. Cellular uptake of siRNA/NP

Silencer[®] FAM (Fluorescein derivative, 5'-carboxyfluorescein)-labeled Negative Control No. 1 siRNA (Cat. # AM4620, Ambion[®]) was incubated with NPs to form complexes as described. For visualization of cellular uptake, mMSCs were seeded in chamber slides (Lab-Tek[®]) at 15,000 cells/cm² one day prior to siRNA/NP treatment. FAM siRNA/NPs (40 nM) were used to treat mMSCs (derived from C57BL/6 mice, purchased from Cyagen[®]) at charge ratios of 4:1 for 24 h. mMSCs were washed 3 times with PBS and fixed with paraformaldehyde (PFA; 4%) for 10 min. mMSCs were then washed with deionized water, and coverslipped with the addition of ProLong[®] Gold Antifade Mounting solution with DAPI (Thermo Fisher Scientific) to prevent quenching. mMSCs were imaged using a Nikon E600 fluorescence microscope. For quantification of cellular uptake, mMSCs were seeded at 8000 cells/cm² in 24-well plates one day prior to treatment. mMSCs were incubated with cell culture media (untreated), free FAM-labeled siRNA (negative control), FAM-labeled siRNA complexed with Lipofectamine 2000 (positive control), as well as FAM-labeled siRNA/NPs at 40 nM and charge ratios of 4:1. After 24 h, mMSC were harvested and analyzed on an Accuri C6 flow cytometer. A total of 5000 cell events were gated for analysis and data was analyzed using FlowJo software.

2.4. Silencing efficiency of siRNA/NP

Wwp1 siRNA and control siRNA were purchased from Ambion[®] (ThermoFisher Scientific). For gene silencing experiments, mMSCs were seeded in 24-well plates at 8000 cells/cm² one day prior to siRNA/NP treatment. Nanoparticles with complexed siRNA (40 nM) were used to treat cells at charge ratios of 1:1–8:1. Cells were also treated with siRNA complexed with Lipofectamine 2000 as a positive control. After 48 h, the cells were washed with PBS (0.5 ml) twice and lysed with TRK Lysis Buffer (OMEGA Bio-Tek). RNA was extracted using Homogenizer mini columns, Hibind mini columns and RNA wash buffers with on-column DNase digestion (Omega Bio-Tek). Extracted RNA was quantified using a NanoVue (GE Healthcare) and RNA concentrations were normalized amongst samples to 50 ng/μl. Then, RNA (2 μL) sample was reverse transcribed to cDNA using an iSCRIPT reverse transcription kit (Bio-Rad) and 2 μl of cDNA was used in RT-PCR reactions using SsoFast Eva-Green Supermix (Bio-Rad) on a CFX96 Real-time PCR Detection System (Bio-Rad). β-actin was used as the housekeeping gene RT-PCR. Primer sequences for *Wwp1* and β-actin are listed in Table S1. *Wwp1* expression was quantified based on the reaction efficiency [29] and normalized to β-actin expression and comparing to untreated cell populations [30].

2.5. Alamar blue metabolic assay for in vitro cytotoxicity of siRNA/NP

MSCs were seeded in 24-well plates at 8000 cells/cm² and treated with polymer/siRNA nanoparticles 24 h later at a variety of charge ratios at 40 nM siRNA doses. Untreated MSCs and Lipofectamine 2000/siRNA were used as negative and positive controls, respectively. After 48 h, MSCs were incubated with a 10% AlamarBlue solution in cell culture medium and incubated for an additional 4 h. Cell culture media was sampled and fluorescence was analyzed at 570 nm/600 nm using a plate reader (Tecan M200 Infinite). Cell viability was calculated by normalizing the fluorescence of media from treated cells to untreated cells.

2.6. Hydrogel formation, swelling, and degradation experiments

PEG-based copolymers poly(ethylene glycol)-*b*-poly(lactide)-*b*-dimethacrylate (PEG_a-*b*-PLA-*b*-DM) were synthesized as previously described by functionalizing linear PEG (Alfa Aesar, MW 2 kDa, 4 kDa, and 10 kDa, a = 46, 91, and 227, respectively) with d,l-lactide and performing microwave-assisted methacrylation using methacrylic anhydride [31,32]. Briefly, PEG was reacted with d,l-lactide at 2:1 (d,l-lactide:PEG) molar ratio by ring-opening polymerization in the presence of tin (II) 2-ethylhexanoate as a catalyst [32]. Subsequent methacrylation of PEG was performed using a domestic microwave as previously described to form PEG-*b*-PLA-*b*-DM [32]. Macromers were isolated via precipitation in ice-cold diethyl ether and dried under vacuum. To determine the number of lactide units and methacrylate functional groups per PEG macromer ¹H NMR analysis was used (Bruker Avance 400 MHz, CDCl₃) [32]. The lactide units were determined to be 2.01 (–CH₂CH₂O– (PEG), 364H, 3.2–3.8 ppm, multiplet; –OCH(CH₃)COO–, 4H/PLA repeat, 5.2–5.3 ppm, multiplet, –OCH(CH₃)COO–, 12H/PLA repeat, 1.4–1.6 ppm, multiplet), with >90% methacrylation ((CH₂=C(CH₃)–, 4H/macromer, 5.6 and 6.3 ppm, singlets; CH₂=C(CH₃)–, 6H/macromer, 1.9 ppm, singlet)). To formulate siRNA/NP loaded hydrogels, PEG-*b*-PLA-*b*-DM (10 wt%) was mixed with the photo-initiator, lithium arylphosphonate (LAP, synthesized as previously described) [33] (0.05 wt%), together with PBS and siRNA/NPs to form a gel solution. PEG gels were prepared via UV photo-polymerization for 10 min at 365 nm and 4 mW/cm² in a teflon mold (6 × 3 mm). siRNA/NP loaded hydrogels were lyophilized overnight and characterized via scanning electron microscope (SEM). To determine the swelling ratio of PEG hydrogels, wet (W_w) and dry (lyophilized) weights (W_d) were measured gravimetrically at various time points over 4 weeks. The swelling ratio (Q) was calculated using $Q = \text{wet weight (W}_w\text{)}/\text{initial dry weight (W}_{di}\text{)}$. The percent mass loss was calculated by $(W_{di} - W_d)/W_{di} \times 100$. The mesh size of hydrogels over the time course of degradation were characterized using the Flory-Rehner equation [34].

2.7. siRNA/NP release study

Silencer[®] FAM-labeled Negative Control No. 1 siRNA (Cat. # AM4620, Ambion[®]) was incubated with NPs to form complexes as previously described which were incorporated into PEG hydrogels at overall siRNA concentration of 1 μM in 40 μl hydrogels. Hydrogels were washed 3 times in PBS to remove loosely associated siRNA/NP at the hydrogel surface, which accounted for approximately 5% of initially loaded siRNA. Hydrogels were placed in PBS on a shaker at 37 °C and protected from light. Releasate was collected every 2–3 days

over hydrogel degradation. Release of siRNA was quantified fluorescently (480 nm/520 nm for FAM-labeled siRNA) using a plate reader (Tecan M200 Infinite).

2.8. Analysis of sustained silencing mediated by hydrogel-released siRNA/NPs in vitro

Wwp1 siRNA and control siRNA were purchased from Ambion® (ThermoFisher Scientific). Mouse MSCs (mMSCs) derived from C57BL/6 mice were cultured at 37 °C and 5% CO₂ in growth media consisting of low glucose Dulbecco's Modified Eagle Medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS), 100 units/ml Penicillin-Streptomycin (Gibco). For gene silencing experiments, mMSCs were seeded in 24-well plates at 8000 cells/cm² one day before siRNA/NP treatment. For *Wwp1* siRNA/NP treated groups, nanoparticles complexed with siRNA were used to treat cells at 40 nM and charge ratios of 4:1. For *Wwp1* siRNA/NP hydrogel treated groups, *Wwp1* siRNA (0.05 nmole) was complexed with NPs, and entrapped in hydrogels (40 µl). Hydrogels were placed in transwell inserts with 0.5 ml cell culture media, to approximate 40 nM siRNA in hydrogel-released complexes over the first 2 days of treatment to emulate the free siRNA/NP treatments. Media was replaced every 3 days. At each time point, cells were washed with 0.5 ml PBS twice and lysed with TRK Lysis Buffer (OMEGA Bio-Tek). RNA extraction, cDNA synthesis, and RT-PCR were performed as described previously. *Wwp1* expression was quantified based on the reaction efficiency and normalized to *β-actin* expression and comparing to untreated cell populations [29].

2.9. Mid-diaphyseal femur fracture model

All animal experiments were approved by the Institutional Animal Care and University Committee of Animal Resources (UCAR). Female 6–8 week old Balb/c mice (approximately 20 g) were purchased from Harlan Laboratories (Indianapolis, IN). After 1 week of acclimatization, mice were anesthetized with ketamine (60 mg/kg) and xylazine (4 mg/kg) intraperitoneally. An 8 mm long skin incision was made on the femur, and blunt dissection of muscle was used to expose the mid-shaft of the femur. Mid-diaphyseal femur fractures were created using a rotary Dremel with a diamond blade attachment. A 25-gauge needle was inserted into the medullary canal of the femur from the distal end. Hydrogels (20 µl) were wrapped around the femur fracture, and the incision was closed with nylon sutures. A Faxitron system (Faxitron X-ray, Wheeling, IL) was used to take X-ray images at the time of surgery and every week following surgery until sacrifice. Buprenorphine (0.05 mg/kg IP) was given 6–12 h prior to fracture and every 6–12 h over 3 days post-fracture.

2.10. In vivo localization of siRNA/NP hydrogels

Cyanine7 (Cy7)-PEG-acrylate (Cy7-PEG-AC) was synthesized using Cy7 NHS ester (C₄₁H₄₈CIN₃O₄, Lumiprobe) and acrylate-PEG-amine (Ac-PEG-NH₂, MW 3.4 kDa, Creative PEGWorks) via carbodiimide chemistry. Cy7-labeled hydrogels were prepared using a combination of PEG-*b*-PLA-*b*-DM and Cy7-PEG-Ac at a molar ratio of 12:1 via photopolymerization, as detailed in previous sections. Unlabeled siRNA/NPs were loaded into Cy7-PEG hydrogels. In another group, Cy5-labeled siRNA (Dharmacon®) complexed with NPs were loaded into unlabeled hydrogels. After fractures, hydrogels (1 nmole siRNA in 20 µl hydrogels) were placed at fracture sites. Local injection of Cy5-siRNA/NPs at fracture sites was used as a control. Localization and release of siRNA/NPs (649 nm/670 nm

for Cy5), as well as hydrogel degradation (750 nm/773 nm for Cy7) were monitored via a XENOGEN/IVIS imaging system (Perki-nElmer) longitudinally. Fur was removed prior to imaging by clippers and depilatory cream to reduce background. IVIS was performed 0, 1, 3, 7, 14, 21 and 28 days after implantation using 2% isoflurane gas as anesthetic. Quantification of hydrogel localization from IVIS imaging was achieved by measuring the total radiant efficiency of regions of interest (right femurs) and normalizing to day 0.

2.11. Healing efficiency of fractures treated with Wwp1 siRNA/NP hydrogels in vivo

Mid-diaphyseal femur fractures were created as described in previous sections. *Wwp1* siRNA complexed with NPs (0.02 nmole siRNA in 20 μ l hydrogels, dose of siRNA 0.013 mg/kg) were loaded into PEG hydrogels and implanted at fracture sites, as previously described. Untreated fractures were used as negative controls. Additional controls included fractures treated with hydrogel alone, free siRNA entrapped in hydrogels, and scrambled siRNA/NP entrapped in hydrogels. Animals were grouped randomly and healing outcomes for groups were scored by blinded observers.

2.11.1. RNA extraction and RT-PCR—Mice were sacrificed on weeks 2 and 3 following surgery. The fracture calluses (8 mm) were carefully dissected to remove muscle and soft tissues and immediately snap frozen in a liquid nitrogen bath. Frozen tissue samples were homogenized using a tissue blender (Bullet Blender[®]) and mRNA was purified via phase separation (Qiagen RNeasy Plus Universal Kit). After cDNA synthesis, RT-PCR was performed to examine the expression level of osteogenic genes (transcription factor runt-related transcription factor 2 (*Runx2*), osteocalcin (*OC*), alkaline phosphatase (*ALP*), and type 1 collagen (*Col1a1*)), as described in previous experimental sections.

2.11.2. Micro-computed tomography—Femurs were harvested at weeks 2 and 3. Femurs were disarticulated from the hip and trimmed to remove excess soft tissue and skin. After 3 days of fixation in 4% neutral buffered formalin (NBF), samples were transferred to PBS and scanned using a Scanco Medical VivaCT 40 at high resolution (10.5 μ m voxel size), with X-ray energy of 55 kVp and intensity of 145 μ amps, and integration time 300 msec. Analyses were performed with Scanco's proprietary software. Each sample was contoured around the external callus and along the edge of the cortical bone. To measure new bone callus volume, fracture contouring was done to exclude cortical bone from all bone space. Bone volume (BV), mineralization density, trabecular thickness, trabecular number, and trabecular bone volume fraction (bone volume (BV)/total volume (TV)) were analyzed in a volume of interest (VOI) including 1 mm of the proximal and distal region of the femur fracture [35]. Samples were kept in PBS at 4 °C before processed for histology.

2.11.3. Histology—After microCT analysis, specimens were decalcified in 10% ethylenediaminetetraacetic acid (EDTA) for 10 days at room temperature. Then, specimens were embedded in paraffin and cut into 5 μ m sections. Alcian blue (blue, glycosaminoglycan/proteoglycan) and Orange G (pink, bone/soft tissue) staining were used to identify soft callus and new bone. Three nonconsecutive sections were obtained from each specimen and imaged at 40 $\times$ magnification using an Olympus VS110 Virtual Microscopy System for whole-slide imaging. Histomorphometric analyses were performed using

Visiopharm software to determine the area of bone, cartilage, and fibrotic tissue (a subtraction of total callus from bone and cartilage tissue) formation by manual tracing [36]. A minimum of six samples was included in each group.

2.11.4. Assessment of torsional biomechanics of fractured femurs—Torsional loading is an effective method for assessing bone strength under nearly pure shear conditions [37]. The proximal and distal ends of harvested femur specimens were cemented into 6.35 mm² aluminum tubes using bone cement prepared to the manufacturers' specifications (DePuy Endurance; Warsaw, IN). A custom jig was used to ensure axial alignment and the maintenance of approximately 7 mm gauge length for all samples. Specimens were submerged in PBS at room temperature for 2 h after potting to allow for rehydration of the tissue and hardening of the bone cement. Samples were mounted on an EnduraTec TestBench™ system (200 N mm torque cell; Bose Corp., Minnetonka, MN) and tested in torsion until failure at a rate of 1°/s to determine ultimate torques [38].

2.12. Statistical analysis

Differences between groups were compared using one-way analysis of variance (ANOVA) or two-way ANOVA with Tukey's or Dunnett's post-hoc testing, as indicated in figure legends. *In vitro* sample sizes were at least 3 per treatment, as indicated in figure legends. For *in vivo* studies, 6–12 mice per condition were used based on an a priori power analysis and preliminary *in vivo* gene expression data. To compare the presence of callus between treatment groups, binomial z distributions were used. A p-value of less than 0.05 was considered significant. Statistics were assessed with GraphPad Prism 6 Software.

3. Results

3.1. Synthesis and characterization of hybrid NP/hydrogel siRNA delivery system

Diblock copolymers (Fig. 1A) composed of dimethylaminoethyl methacrylate (DMAEMA), propylacrylic acid (PAA), and butyl methacrylate (BMA) pDMAEMA-*b*-p(DMAEMA-*co*-PAA-*co*-BMA) have shown successful siRNA delivery to a variety of cell types [25,26,39–41]. These polymers, which are synthesized using reversible addition-fragmentation chain transfer (RAFT) polymerization, as detailed previously [25], self-assemble into NPs of 27 nm (Table 1) under aqueous conditions with positive zeta potential (14 mV, Table 1) to enable electrostatic interaction with siRNA (Fig. 1B). Diblock molecular weight (Mn), block ratio, and composition (diblock 1st and 2nd block molecular weights (Mn) were 21,900 g/mol and 12,600 g/mol, composition: DMAEMA:PAA:BMA = 23:30:47, Table 1) were chosen from a library of polymers screened for maximized mMSC gene silencing efficiency with minimal cytotoxicity [28]. Fluorescent microscopy of mouse MSCs (mMSC) showed excellent cellular uptake of FAM-labeled siRNA/NPs 24 h after treatment (Fig. 1F). siRNA delivered by NPs showed significant higher median fluorescent intensity (MFI) compared to untreated and free siRNA treated MSCs, with comparable MFI to Lipofectamine 2000, which is a commercially available positive control for siRNA delivery (Fig. 1G).

To demonstrate the therapeutic potential of this NP platform for bone regeneration, siRNA targeting *Wwp1* was used to form NP complexes. Singular doses of *Wwp1* siRNA/NPs

resulted in significant mMSC gene knockdown without compromising cell viability *in vitro* (Fig. S1). These data are consistent with previous studies using similar NPs that showed excellent siRNA delivery and gene knockdown [25,27,28], which is attributed to siRNA protection from degradation, improved cellular uptake, and excellent cytoplasmic localization due to pH-responsive endosomolytic behaviors.

To utilize PEG hydrogels to sustain and control the release of siRNA/NP, hydrophilic PEG chains were first modified with hydrolytically degradable poly(lactic acid) (PLA) functionalities. These PEG-PLA chains were then functionalized with methacrylate groups to allow for photopolymerization-mediated hydrogel formation and siRNA/NP entrapment (Fig. 1C). In this hybrid NP/ hydrogel delivery system, NPs protect siRNA and increase siRNA delivery efficiency, while PEG hydrogels provide localized and sustained siRNA/NP delivery through hydrolytic degradation of crosslinks. Briefly, PEG-*b*-PLA-*b*-DM macromers, siRNA/NP complexes, and lithium arylphosphonate (LAP) photoinitiator were combined in phosphate buffered saline (PBS). Hydrogels were formed via exposure of solutions to low intensity, long-wave UV light. NPs were uniformly entrapped within hydrogels without aggregation, as depicted via scanning electron microscopy (SEM) (Fig. 1E). siRNA/NP release rate and hydrogel persistence is controlled via hydrogel mesh size, which changes temporally due to crosslinker degradation. As shown in Fig. 1H, lactide-containing crosslinks degrade over 4 weeks, resulting in increases in overall mesh size from 10 to 70 nm. Based on the size of NPs (27 nm), NPs release was initially significantly hindered within hydrogels. However, due to degradation, hydrogels loaded with FAM-labeled siRNA/NPs showed a sustained release of siRNA/NPs over the time course of hydrogel degradation, which was ~4 weeks (Fig. 1I). The release of siRNA/NPs was fit to a 1st order release model (dotted line) and the release rate constant, k' , was calculated to be 0.21 day^{-1} , which is approximately 7-fold slower than the release rate of free siRNA from the hydrogel ($k' = 1.5 \text{ day}^{-1}$), indicating controlled release behavior using the NP/hydrogel system. Hydrogel release rate can be tuned by changing initial hydrogel mesh size by varying PEG molecular weight (Fig. S2), as higher PEG molecular weight results in greater distance between crosslinks, thereby increases release rate. Hydrogels were formed from 2 kDa, 4 kDa, and 10 kDa PEG; 4 kDa provided more substantial release of siRNA/NP compared to 2 kDa with more prolonged release compared to gels formed from 10 kDa. Therefore, 4 kDa hydrogels were chosen for gene silencing studies *in vitro* and *in vivo*. Though not used here, release rate of siRNA/NP can be further tuned by weight percentage [42,43] or through incorporation of alternative degradable bonds within hydrogel crosslinks (Fig. S3) [44].

3.2. Sustained gene silencing ability of siRNA/NP released from hydrogels in vitro

To show the hybrid NP/hydrogel system is capable of sustained siRNA-mediated gene regulation, mMSCs were treated *in vitro* with hydrogel-released siRNA/NPs via transwell plates. Quantitative real-time PCR (RT-PCR) analysis was performed to assess the expression level of *Wwp1* and genes associated with osteogenesis and bone formation over time. mMSCs treated with a single dose of siRNA/NP on day 1, as well as untreated mMSCs cultured in normal and osteogenic media were used as controls. A single dose of *Wwp1* siRNA/NPs provided transient *Wwp1* knockdown of 53% at day 3 (Fig. 2A). By day 7,

Wwp1 expression recovered to untreated control cell levels. For cells treated with *Wwp1* siRNA/NP releasing hydrogels, the knockdown of *Wwp1* lasted for 12 days. At day 3, *Wwp1* expression was reduced to 19% of mMSC controls. At day 7, siRNA/NP hydrogel treated cells maintained a low level of *Wwp1* expression (20%). By day 10, *Wwp1* expression recovered to 32% for siRNA/NP hydrogel treated cells and 77% for cells treated with a single dose of siRNA/NPs. By day 12, *Wwp1* expression was further recovered to 51% for siRNA/NP-hydrogel treated cells, which was significantly different from negative controls. Sustained knockdown was consistent with the time scale of siRNA/NP release, as shown in Fig. 1I.

The expression levels of osteogenic genes after treated with *Wwp1* siRNA/NP were investigated. The expression of osteogenic transcription factor runt-related transcription factor 2 (*Runx2*) [45], which is negatively regulated by *Wwp1*, along with other downstream of *Runx2*, osteocalcin (*OC*), alkaline phosphatase (*ALP*), and type 1 collagen (*Colla1*), peaked at day 7 after treatment with a single dose of siRNA/NP or siRNA/NP releasing hydrogels, with hydrogel/NP-treated cells showing significant higher expression (Fig. 2B). At day 12, the expression of *Runx2* reduced in untreated and siRNA/NP treated cells, whereas hydrogel/NP-treated cells still showed 3-fold higher expression compared to day 1. Expression levels of the osteoblast differentiation markers *ALP* (Fig. 2C), *Colla1* (Fig. 2D), and *OC* (Fig. 2E) were also elevated. A single dose of siRNA/ NPs was able to induce upregulation of *ALP* and *OC* at day 7. By day 10, however, *ALP* and *OC* were reduced, which was in agreement with transient *Wwp1* knockdown. In contrast, expression of *ALP*, *OC*, and *Colla1* peaked later and were sustained until day 12 in hydrogel treated cells.

3.3. Localization of siRNA/NP hydrogels and their gene silencing efficiency in vivo

Having established that PEG hydrogels can control the release of siRNA/NPs *in vitro*, resulting in sustained gene regulation, controlled release behavior was further investigated *in vivo*. Cy5-labeled siRNA/NPs and non-fluorescent siRNA/NP were loaded into non-fluorescent or Cy7-labeled hydrogels, respectively, to independently study the localization of both siRNA and hydrogels. siRNA/NP loaded hydrogels were implanted at mid-diaphyseal femur fractures. Untreated fractures and direct injection of Cy5-labeled siRNA/NPs at the fracture site were used as controls. Following hydrogel implantation, mice were periodically anesthetized and imaged using *in vivo* imaging (Xenogen IVIS) (Fig. 3A). Injections of free siRNA/NPs showed rapid dispersal and clearance from the fracture site by day 3 (Fig. 3A), with a $t_{1/2}$ of 17 h (Fig. 3B). Similar to the *in vitro* data, siRNA/NP from hydrogels persisted at fractures for more than 3 weeks with $t_{1/2}$ of 3.9 days. The hydrogel alone was localized for approximately 28 days with $t_{1/2}$ of 3.5 days, which is statistically equivalent to the siRNA/NP signal, suggesting colocalization of siRNA/NPs within hydrogels. The results indicated that NP/hydrogel depots are able to maintain high local concentration of siRNA in the surrounding tissues over an extended period.

To evaluate the gene silencing efficiency of siRNA delivered by NP/hydrogel, fractures were treated with *Wwp1* siRNA/NP-loaded hydrogels. Quantitative real-time PCR analysis was performed to determine the expression of genes associated with bone formation in the callus tissue. Untreated fractures, fractures treated with hydrogel only (without siRNA/NP), *Wwp1*

siRNA (no NP) entrapped hydrogels, and scrambled siRNA/NP releasing hydrogels, direct injection of *Wwp1* siRNA/NPs, as well as unfractured mice were studied as controls. Two weeks after femur fractures and hydrogel implantation, *Wwp1* gene expression level in the *Wwp1* siRNA/NP hydrogel treated fractures was reduced to 23% compared to untreated fractures (Fig. 3C). This reduction in expression is significant compared to all control groups except for unfractured mice, indicating that knockdown of *Wwp1* is specific to NP-mediated delivery of *Wwp1* siRNA and not due to effects of hydrogels, NP, or nonspecific siRNA. Downstream expression of osteogenic genes *Runx2* (Fig. 3D), *OC* (Fig. 3E), *ALP* (Fig. 3F), and *Col1a1* (Fig. 3G) were all upregulated in *Wwp1* siRNA/NP hydrogel group compared to untreated and no fracture groups, corresponding to the decrease in *Wwp1* expression. *OC* expression was significantly upregulated compared to all negative control groups, including scrambled siRNA/NP hydrogel; and scrambled siRNA/NP hydrogel-treated fractures showed no significant changes in *Wwp1* expression and downstream gene expression compared to any negative control group. Direct injection of *Wwp1* siRNA/NP was not sufficient to induce effective gene silencing and osteogenic gene expression by week 2, which is in agreement with the rapid dispersal observed in Fig. 3A. To examine if gene knockdown induces *Wwp1* protein knockdown *in vivo*, immunostaining for *Wwp1* was performed in naïve bone, untreated fractures, or fractures with various treatments (Fig. S4). Two weeks after fracture, untreated controls showed strong expression of *Wwp1* at fracture soft callus, while *Wwp1* siRNA/NP hydrogel treated fractures showed significant reduction in *Wwp1* knockdown compared to other treatments. Taken together, these data indicate the NP-mediated specific knockdown of *Wwp1* resulted in a statistically robust osteogenic effect.

3.4. Fracture healing efficiency *in vivo*

Fracture healing is a complex and highly orchestrated biological process involving multiple pathways [46–48]. Recruited MSCs differentiate into chondrocytes and osteoblasts, both of which secrete collagen-rich matrix that calcifies and bridges the fracture site [49]. To determine whether *Wwp1* siRNA/NP hydrogels enhance fracture healing, radiographic imaging was performed at 14 and 21 days post-fracture (Fig. 4A, Table S2). Calcified callus was apparent 2 weeks after fracture in 4 of 12 mice treated with *Wwp1* siRNA/NP hydrogels, compared with only 1 of 12 untreated mice. By week 3, only 6 of 12 untreated mice had radiographic evidence of callus formation, compared to 11 of 12 *Wwp1* siRNA/NP hydrogel treated mice. To further investigate how *Wwp1* siRNA/NP hydrogels alter fracture healing, bone callus formation was quantified using microcomputed tomography (μ CT) 2 and 3 weeks after hydrogel implantation (Fig. 4B). *Wwp1* siRNA/NP hydrogel treated fractures exhibited callus formation at week 2 and formation of complete bridging bone collars by week 3. In contrast, untreated controls exhibited ~3.7-fold decreased bone volume compared to *Wwp1* siRNA/NP hydrogel treated fractures. In addition, callus formation in control groups, including untreated fractures, failed to form completely bridged callus by week 3. Quantitatively, *Wwp1* siRNA/ NP hydrogel treated fractures were the only group that showed significant increases in bone volume and bone volume fraction (BV, and BV/TV) compared to no treatment controls (Fig. 4C).

Histology demonstrated accelerated fracture healing in *Wwp1* siRNA/NP hydrogel treatment compared with untreated mice (Fig. 5A). On day 14 following fracture, the *Wwp1* siRNA/NP hydrogel group had increased callus area and cartilage formation relative to untreated mice (Fig. 5A (e) and Fig. 5B). By day 21, *Wwp1* siRNA/NP hydrogel treated mice had significantly decreased unmineralized cartilage and increased bone area (Fig. 5B and C). Hard callus and bridging bone was also observed in *Wwp1* siRNA/ NP hydrogel treated mice and femurs started remodeling and returning to normal geometries (Fig. 5A (k)). In contrast, soft callus with no bridging is observed in control treatment groups (Fig. 5A (g–l)). In addition, the amount of fibrotic tissue in *Wwp1* siRNA/NP hydrogel treated fractures was significantly lower than that in untreated mice (Fig. 5D). Overall, histological assessments indicated that *Wwp1* siRNA/NP hydrogel treated fractures exhibited earlier endochondral ossification, faster healing, accelerated bone formation and mineralization, and less fibrotic tissue formation, which are consistent with the results from radiographic and μ CT analysis.

Torsion testing was performed to assess the biomechanical stability of healing femurs. Yield torque is defined as the torque at which bone experiences plastic deformation while maximum torque was defined as when fractures occur. *Wwp1* siRNA/NP hydrogel treated fractures exhibited yield torque (Fig. 6A) and maximum torque values (Fig. 6B) that were 2.4– and 2.9– fold higher than untreated fractures, respectively, and both are significantly increased compared to scrambled siRNA/NP hydrogel treated fractures, indicating that the enhancement in bone strength is due to the specific gene silencing mediated and subsequent enhancement of osteogenesis by *Wwp1* siRNA/NP hydrogels.

4. Discussion

siRNA-based therapies have been explored for a variety of diseases, such as cancer and genetic disorders [4], and only recently have gained attention for use in regenerative medicine applications [9]. However, siRNA has multiple unfavorable physicochemical characteristics hampering effective *in vivo* delivery [50]. Systemic administration of naked siRNA molecules resulted in rapid serum nucleases degradation and short plasma half-lives of <10 min [51]. In addition, siRNA exhibits low cellular internalization due to high molecular weight and anionic characteristics, which precludes association with cell membranes for uptake [25]. Furthermore, siRNA must localize to the cytosol once taken up, where integration into the RNA-induced silencing complex (RISC) and subsequent mRNA degradation occurs [3,4]. Research to date has largely focused on development of carriers that protect siRNA from nucleases and enhance cellular uptake and endo-lysosomal escape [52]. Carriers typically bind to siRNA electrostatically or covalently, forming nanoparticles. Upon systemic delivery, liver dominates siRNA/carrier biodistribution due to reticuloendothelial system (RES) function (e.g., macrophages and liver Kupffer cells) [53], resulting in highly effective siRNA-mediated liver disease modification and several ongoing clinical trials [54]. However, as a consequence of liver accumulation, high doses of systemically administered siRNA are required to reach therapeutic levels at other tissues, such as bone, posing a risk for adverse off-target effects [55]. Though modification of delivery systems with targeting ligands has been attempted to alter biodistribution [4,11,41,55,56], liver still remains as a major organ for siRNA biodistribution. For example,

bone targeting functionalities have been introduced to increase bone localization from approximately 1 to 8% of injected dose, significant siRNA accumulation was still observed in liver (>50% of injected dose) [11,55,56].

As an alternative to systemic delivery, local injection of siRNA has been demonstrated for lung [57], brain [58], spinal cord [59], and eyes [60]. Additionally, we have shown successful local siRNA delivery to the salivary gland to achieve therapeutic gene silencing *in vivo* [40,61]. However, a major drawback of this approach for non-encapsulated organs with readily accessible duct systems (e.g., salivary gland, lung) and acute therapeutic requirements is rapid dispersal from the delivery site, which limits drug efficacy and elicits off-target effects in healthy tissues [18]. Rapid dispersal from fracture sites (Fig. 3A) following direct injection was observed, which is unlikely to affect healing (Figs. 4–6). To achieve knockdown over timeframes commensurate with tissue regeneration, local injection may require multiple siRNA/carrier injections, a feature further complicated by exhaustion of silencing efficiency within approximately 7 days in rapidly proliferating cells (also shown in Fig. 2A) [25].

To realize tissue-specific, sustained delivery of siRNA, we developed a nanoparticle/hydrogel hybrid system, which offers the advantages of tissue-specific localization and sustained release of siRNA over 30 days, which is consistent with bone healing. Meanwhile, local delivery at the target tissue significantly reduces the overall siRNA dose and avoids off-target effects. The aforementioned bone-targeted siRNA delivery system requires doses of 3.75 mg/kg to be effective, of which >50% accumulates in the liver [11]. Nanotoxicity and immunogenicity remain major concerns for clinical translation and can inflict significant unintended side effects to tissues and organs [62]. There is evidence that cellular uptake of siRNA can trigger innate immune responses and affect cell functions [4]. It has also been reported that high levels of hepatotoxicity can result from systemic injections of cationic siRNA formulations [63]. In contrast, the NP/hydrogel hybrid system developed herein demonstrated effective gene knockdown at local tissue with an overall dose of 0.013 mg/kg, which is approximately 500-fold lower than doses typically used systemically [11,55,56].

To achieve controlled delivery, siRNA complexed nanoparticles (siRNA/NP) were entrapped within poly(ethylene glycol)-poly(lactic acid)-dimethacrylate (PEG-PLA-DM) hydrogels. While hydrogels can be formed from both natural [64–66] and synthetic materials [67–69], poly(ethylene glycol) (PEG)-based hydrogels are synthetically versatile, degradable, biocompatible, and exhibit tissue-like properties [70–73]. PEG hydrogels are also FDA approved for multiple applications [15] and are one of the most successful synthetic hydrogels for tissue engineering applications [31,32,70–72,74–76]. Here, PEG crosslinks form a nano-scale mesh that enables siRNA/NP loading; hydrolytically degradable ester bonds within PLA control hydrogel degradation rates [2] and release of embedded siRNA/NPs as the mesh size increases. As shown in Fig. S4A, mesh size of hydrogels increases with increasing PEG molecular weight, resulting in faster release kinetics of siRNA/NPs (Fig. S4B). Though not explored here, hydrogel weight percent and/or number of PLA repeats can be tuned to match desired dose and longevity of siRNA/NP release [42–44,75]. Finally, hydrogels can be non-invasively polymerized *in situ* [71,77] and, as they degrade hydrolytically, a secondary retrieval of hydrogel materials is unnecessary.

For tissue regeneration applications that require localized and sustained delivery of siRNA, the developed system achieved sustained delivery of bioactive siRNA to the specific injury site (e.g., 30 days, over the typical time course of bone healing). However, this approach released approximately 50% of siRNA/NP over the first 7 days after implantation (Fig. 1I), potentially reducing siRNA efficacy during later stages of healing. Though PEG molecular weight, PLA repeats, and hydrogel weight percentage can be altered to achieve better control, enzymatically-degradable crosslinkers or tethers could also be used. Previously, matrix metalloproteinase (MMP) degradable crosslinkers and tethers have been incorporated into PEG hydrogels, resulting in MMP-responsive and sustained release of peptide drugs *in vivo* over several hours to 10 days [71,76]. For orthopedic applications, release could be controlled by taking advantage of differentially expressed enzymes during the healing process. For example, peptide linkers that are specifically degraded by cathepsin K, a protease secreted by osteoclasts during bone remodeling, could be incorporated into PEG hydrogels to provide siRNA release only at the culmination of healing [78].

Here, we demonstrated the ability of a hybrid NP/hydrogel system to deliver therapeutic siRNA to expedite fracture healing. Fracture healing is a complex process in which bone injury is followed by inflammation, recruitment and differentiation of MSCs, and bone remodeling [79]. The traditional use of bone morphogenetic proteins (BMPs), the only drugs approved clinically for treating fractures, is limited by numerous complications such as inflammation and ectopic bone formation [48,80–82]. A recent study found that local delivery of BMP-2 to bone defects increased bone formation in a dose-dependent manner, however significantly decreased bone mineral density (BMD) and trabecular number was observed, indicating compromised bone microarchitecture and poor bone quality [82]. RNA interference (RNAi), an intracellular mechanism that regulates gene expression through precise gene silencing post-transcriptionally, has great potential as an alternative in bone regeneration. The regenerative siRNA used herein, which targets *Wwp1*, negatively regulates osteogenic precursors through modification of key positive regulators of osteogenesis including *Runx2* and MSC migration regulator CXC chemokine receptor 4 (CXCR4) [24,83]. Consistent with the global knockout model, we observed significantly increased *Runx2* expression both *in vitro* and *in vivo* after *Wwp1* siRNA treatment. Although *Wwp1* siRNA/NP hydrogels improved mineralized bone volume compared to untreated and unfractured control groups, it was not significantly increased over scrambled-siRNA/NP delivery (Fig. 4C). A recent study in our lab using RNA sequencing (RNAseq) has shown that NP-mediated delivery of negative control siRNA to human MSCs *in vitro* resulted in significant upregulation of innate immune signaling pathways, likely through siRNA stimulation of toll-like receptors [27]. This could explain the mild, yet statistically insignificant healing effect of the scrambled siRNA/NP hydrogel group, as upregulation of the innate immune response has been shown to improve fracture healing [84]. Nevertheless, significant bone formation (Fig. 5), accelerated bone remodeling (Fig. 5), and overall bone strength (Fig. 6) were found to be significantly increased compared to both untreated and scrambled siRNA/NP hydrogel controls. Ultimately, specific gene silencing of *Wwp1* conferred by siRNA/NP enhanced mechanical properties, which is the ultimate metric for bone healing.

The hydrogel system was designed to localize the delivery of *Wwp1* siRNA/NP to fractures to reduce off-target delivery. However, the fracture healing microenvironment, which is composed of multiple cell types, including MSCs, osteoblasts, osteoclasts, macrophages, etc. [79], was exposed to *Wwp1* siRNA/NP as well. The function of *Wwp1* in these heterogeneous population is not fully understood. However, enhanced osteogenesis of MSCs was observed in *Wwp1*^{-/-} mice without adversely affecting osteoclast formation [24]. Thus, our approach is not expected to initiate adverse effects on non-therapeutic cells, such as osteoclasts or macrophages. Nonetheless, careful interrogation of this possibility is warranted in future studies [85].

The study herein constitutes the first development and use of sustained and localized release approach for siRNA/NP to enhance fracture healing and demonstrates efficiency in a clinical-relevant model. As an effective approach to translate identified gene targets to clinical settings, this hybrid NP/hydrogel system is versatile due to its ability to deliver virtually any siRNA. This approach can also deliver other therapeutic structurally similar nucleic acids such as miRNAs, which are endogenous small RNAs that act as gene repressors post-transcriptionally [86]. For example, miR-2861 [87] and miR-26a [88] have been identified to enhance osteogenesis and bone formation. Another advantage of this approach is noninvasive, injectable introduction of hydrogels arthroscopically, circumventing costly and damaging surgical placement.

5. Conclusions

PEG hydrogels were designed for siRNA/NPs delivery to fracture sites in a sustained and localized manner. Target knockdown of *Wwp1* using siRNA/NPs hydrogels showed significantly accelerated healing and increases in bone formation. The development of this hydrogel/NP siRNA delivery system has outstanding therapeutic promise for fracture healing and beyond, as many cell functions can be altered to achieve tissue regeneration and healing, including proliferation, differentiation, and matrix production. Importantly, without modifying the chemistry of NPs or hydrogels, different therapeutic siRNAs can be introduced, resulting in nearly endless possibilities for this therapeutic biomaterials approach.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. The hybrid nanoparticle (NP)/hydrogel siRNA delivery system provides a sustained gene silencing platform

(A) Composition of siRNA delivery diblock copolymers. Diblock copolymers were composed of a cationic (blue) dimethylaminoethyl methacrylate (DMAEMA) block (m~139) and endosomolytic (black) blocks of DMAEMA, propylacrylic acid (PAA), and butyl methacrylate (BMA) (n~91). (B) Positively charged NPs and negatively charged siRNA molecules formed complexes via electrostatic interactions. (C) Chemical structure of Poly(ethylene glycol)-*b*-poly(lactide)-*b*-dimethacrylate (PEG_a-*b*-PLA_b-*b*-DM, a = 91, b = 2 for 4 kDa PEG_a-*b*-PLA_b-*b*-DM macromers). (D) siRNA/NP complexes were incorporated into degradable PEG-based hydrogels with controlled release characteristics. Hydrogels provided localized and sustained release of siRNA/NP due to crosslink degradation and subsequent increase in mesh size. (E) NPs with size of 27 ± 3 nm displayed spherical morphology in PEG hydrogels using SEM. (F) FAM-labeled siRNA was complexed with diblock nanoparticles, resulting in robust cellular uptake in mMSCs 24 h after treatment. (G) Quantification of median fluorescent intensity (MFI) of MSCs treated with free FAM-siRNA, and FAM-siRNA complexed with Lipofectamine 2000 (positive control) or NPs. Mean ± STDEV, n = 3, Statistical significance was evaluated at a level of P < 0.05 using two-way ANOVA with Dunnett's post-hoc analysis with * and # indicating significance of siRNA/NP compared to no treatment and free siRNA, respectively. (H) Swelling and degradation behavior obtained from 4 kDa PEG hydrogels over 4 weeks. Mean ± STDEV, n = 3. (I) Temporal release profile of free siRNA and siRNA/NPs loaded in PEG hydrogels. Mean ± STDEV, n = 3. Dotted lines indicate curve fit to a 1st order release model. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 2. Hydrogel sustained knockdown via released siRNA/NPs

A transwell assay was used to validate the sustained activity of siRNA/NP released from hydrogels. mMSCs were seeded in 24 well plates and *Wwp1* siRNA/NP loaded hydrogels were placed in transwell inserts with 8 μ m pore size. Media was changed every 3 days. Gene expression of *Wwp1* was analyzed using RT-PCR at 3, 7, 10, and 12 days after treatments. Sustained knockdown of (A) *Wwp1* in mMSCs treated with *Wwp1* siRNA/NP releasing hydrogels was observed. Downstream osteogenesis related gene expression, including (B) osteogenic transcription factor *Runx2*, (C) osteoblast markers *ALP*, (D) *Col1a1* and (E) *OC* were analyzed using RT-PCR at day 3, 7, 10, and 12 days after treatments. Mean $\pm$ STDEV, n = 3. Statistical significance was evaluated at a level of P < 0.05 using two-way ANOVA with Dunnett's post-hoc analysis with *, #, and \$ indicating significance of hydrogel treatments compared to MSC media, osteogenic media, and osteogenic media + *Wwp1* siRNA/NP, respectively.

Fig. 3. Long term localization of siRNA/NP and hydrogel at fracture site results in local and sustained gene knockdown

(A) Representative IVIS images of Cy5-siRNA/NP loaded hydrogels and Cy7-labeled hydrogels at right femur fractures over time. 1 nmole of Cy5-siRNA was injected at fracture sites (free siRNA/NPs) or embedded into 20 μ l 4 kDa PEG hydrogels and implanted at fractures. Hydrogel prepared using 4 kDa Cy7-labeled PEG was also implanted at fractures. siRNA and hydrogels were co-localized at fractures for ~21 days, whereas free siRNA/NP dispersed from injection sites rapidly. (B) Quantification of siRNA/NP and hydrogel localization from IVIS imaging. Data showed the total radiant efficiency of drawn region of interest (ROI) in IVIS images normalized to day 0. Mean $\pm$ STDEV, $n = 6$. (C) Fold change gene expression of *Wwp1* 14 days after treatment of siRNA hydrogels (0.02 nmole siRNA in 20 μ l hydrogels, dose of siRNA 0.013 mg/kg). (D) Fold change gene expression of *Runx2*, (E) *OC*, (F) *ALP*, and (G) *Col1a1* 14 days after treatments. Mean $\pm$ STDEV, $n = 8$. For (C) to (G), statistical significance was evaluated using one-way ANOVA with Tukey's post-hoc analysis. *, #, \$, %, &, and δ $p < 0.05$ versus no treatment, hydrogel only, *Wwp1* siRNA + gel, scrambled siRNA/NP + gel, *Wwp1* siRNA/NP injection, and no fracture, respectively.

Fig. 4. Accelerated callus formation is observed in fractures treated with *Wwp1* siRNA/NP hydrogels

(A) Radiographs taken at 14 and 21 days showed callus formation and fracture healing.

Diaphyseal fractures were created in wild-type mice as described in Methods.

Representative radiographs of untreated fractures (a and g) and fractures treated with hydrogel only (b and h), naked siRNA-loaded hydrogels (c and i), scrambled siRNA/NP loaded hydrogels (d and j), *Wwp1* siRNA/NP loaded hydrogels (e and k), and *Wwp1* siRNA/NP injection (f and l) obtained on week 2 (a–f) and week 3 (f–l) after fracture are shown. Of note is the presence of callus at week 2 and bony union at week 3 in *Wwp1* siRNA/NP hydrogel treated mice. Scale bar = 3 mm. (B) Micro-computed tomography (μCT) scans revealed increased *in vivo* bone callus formation with siRNA/NP hydrogel treatments. μCT scans were taken 2 and 3 weeks post-hydrogel implantation to assess *in vivo* bone callus formation (intact and cut views). (C) Subsequent quantification showed *Wwp1* siRNA/NP loaded hydrogels significantly increased bone callus volume (i) and (ii) bone volume fraction (bone volume/total volume) as compared to transplantation of other hydrogel systems, as well as untreated controls. Mean ± STDEV, n = 6. Statistical significance was evaluated using two-way ANOVA with Tukey's post-hoc analysis. *, #, \$, %, and & p < 0.05 vs no treatment, hydrogel only, *Wwp1* siRNA + gel, scrambled siRNA/NP + gel, and *Wwp1* siRNA/NP injection (at each time point), respectively.

Fig. 5. Sustained *Wwp1* silencing resulted in expedited endochondral bone formation and mineralization

(A) Histological analysis of fractured femur sections was performed using alcian blue (blue; glycosaminoglycans and proteoglycans) and orange G staining (pink; bone and surrounding soft tissue). Untreated (a and g), and fractures treated with hydrogel only (b and h), naked *Wwp1* siRNA-loaded hydrogel (c and i), scrambled siRNA/NP loaded hydrogel (d and j), *Wwp1* siRNA/NP loaded hydrogels (e and k), and *Wwp1* siRNA/NP injection (f and l) were obtained on week 2 (a–f) and week 3 (g–l) after fracture. The percentage area of (B) unmineralized cartilage, (C) bone, and (D) fibrotic tissue were quantified using Visiopharm software. Mean $\pm$ STDEV, $n = 6$. Statistical significance was evaluated using two-way ANOVA with Dunnett's post-hoc analysis. *, #, \$, %, and & $p < 0.05$ vs no treatment, hydrogel only, *Wwp1* siRNA + gel, scrambled siRNA/NP + gel, and *Wwp1* siRNA/NP

injection (at each time point), respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 6. Fractures treated with *Wwp1* siRNA/NP releasing hydrogels exhibited increased biomechanical strength

(A) Yield torque and (B) maximum torsion strength of *Wwp1* siRNA/NP hydrogel treated fractures was significantly increased over untreated controls. Mean $\pm$ STDEV, $n = 6$. Statistical significance was evaluated using one-way ANOVA with Tukey's post-hoc analysis. *, #, \$, %, & and δ $p < 0.05$ vs no treatment, hydrogel only, *Wwp1* siRNA + gel, scrambled siRNA/NP + gel, *Wwp1* siRNA/NP injection, and no fracture, respectively.

Table 1

Characterization of polymer and NP.

Polymer 1st block			Polymer Diblock			NP characterization		
Mn ^a (g/mol)	Mw ^b (g/mol)	PDI (Mw/Mn)	Mn ^a (g/mol)	Mw ^b (g/mol)	PDI (Mw/Mn)	Diameter (nm)	PDI	Zeta Potential (mV)
21,900	22,500	1.03	34,500	35,000	1.01	27 ± 3	0.18	14 ± 1

^aNumber average molecular weight, Mn.^bWeight average molecular weight, Mw.