SPECIAL FOCUS: RNA THERAPEUTICS FOR TISSUE ENGINEERING*

Silk Biomaterials-Mediated miRNA Functionalized Orthopedic Devices

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Silk-based bioresorbable medical devices, such as screws, plates, and rods, have been under investigation due to their promising properties for orthopedic repairs. Options to functionalize these new devices for enhanced control of bone regeneration would also exploit the compatible processing methods used to generate the devices. MicroRNAs are important regulators of bone maintenance and formation, and miRNA-based therapeutics have the potential to aid bone repair, utilizing a transient therapeutic approach with local bioactivity. We hypothesized that silk-based orthopedic devices could be used for the local delivery of miRNAs, using anti-sense miR-214 (AS-miR-214), to inhibit endogenous expression of osteoinductive antagonist and thereby supporting the upregulation of osteoinductive target molecules activating transcription factor 4 (ATF4) and Osterix (Osx). AS-miR-214 silk devices, prepared using surface coating, demonstrated continuous release of miRNA inhibitors up to 7 days *in vitro*. Additionally, human mesenchymal stem cells seeded on AS-miR-214 silk films expressed higher levels of osteogenic genes ATF4, Osx, Runx2, and Osteocalcin. Interestingly, these cells exhibited lower cell viability and DNA content over 21 days. Conversely, the cells demonstrated significantly higher levels of alkaline phosphatase expression and calcium deposition compared with cells seeded on silk films with nontargeting miRNA controls. The study demonstrated that the silk-based orthopedic devices, in conjunction with bioactive miRNA-based therapeutics, may serve as a novel system for localized bone tissue engineering, enhancing osteogenesis at the implant interface while avoiding detrimental systematic side effects.

Keywords: silk, miRNA, orthopedic, biomimetic, nonviral transfection

Introduction

ORTHOPEDIC SILK MEDICAL devices, prepared from silkworm silk derived from *Bombyx mori*, represent new and promising candidates for bone repair and tissue regeneration due to their mechanical properties, low immunological responses, biodegradability, and ease of functionalization.^{1–6}

For bone fracture fixation, as well as guided tissue formation, successful engineered biomaterials must be biocompatible and resorbable, present an environment with appropriate cues for cell differentiation and deposition of new matrix, provide appropriate mechanical properties, and exhibit osteoconductivity. To this end, we recently developed a variety of chemical, structural, and biomechanical modifications of silk to improve function for bone tissue fracture repairs.^{1,2,4–6} These studies have focused on improving osteoconductivity through modifications with bioactive molecules to facilitate bone formation and for mechanical reinforcement.

MicroRNAs (miRNAs, miRs) are important regulators of bone maintenance and have emerged as powerful new

therapeutic molecules.^{7–11} miRNAs are short, noncoding RNAs that can base pair with target mRNAs, to decrease stability and translatability of the mRNA target genes.¹² Since a single miRNA can target several mRNAs, frequently within the same or correlated pathways, this approach has the potential to broadly affect cell growth and differentiation. Chemically modified small RNAs can act as microRNA inhibitors or mimics. Indeed, there have been excellent advances with regard to the design of chemical modifications of RNAs that increase stability and efficacy, while decreasing immunogenicity.¹³ To this end, many emerging areas of regenerative medicine have recently explored the role of a series of miRNAs by inhibiting or mimicking their function, sparking interest in therapeutic use for tissue repair. When transiently transfected into cells, these RNAs can remain active for up to a week or longer, increasing the probability of a sustained effect, with a relatively small initial dose of RNA.^{14,15} Noteworthy, miRNA-based therapeutics should not be viewed as “gene therapy”; they are small RNAs that do not need to enter the nucleus for

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activity. Moreover, the small size of miRNAs is likely to improve cellular uptake. Importantly, therapeutic small RNAs will not integrate into DNA, thus eliminating concerns about genomic alterations.¹⁶ Developing functional devices that better simulate the microenvironment cells are subjected *in vivo*; by exposing cells to miRNA silk-based implants could provide biological cues advantageous to accelerated bone healing and tissue integration. Furthermore, localized release will avoid concerns associated with systemic side effects.

Recent studies have identified miRNAs as master regulators for bone maintenance and repair.¹² Various miRNAs have been demonstrated as key regulators of bone formation and maintenance with substantial control of osteoblast^{17–23} and osteoclast^{24–31} differentiation and functions. Recently, biomaterials functionalized with miRNA to regulate skeletal tissues, aimed at upregulating extracellular matrix production,^{15,32} and healing critical size bone defects³³ have been reported. Previously used biomaterials include collagen hydrogels,³⁴ polylactic acid microspheres,^{19,21} as well as electrospun gelatin nanofibers.¹⁵ Hence, the use of miRNA therapeutics to enhance silk-based orthopedic devices may serve as a novel platform for functionalization.

miR-214 is highly conserved in vertebrates and is encoded within a larger noncoding RNA, Dnm3 opposite strand (*Dnm3os*).³⁵ Dnm3os has emerged as a cluster of miRNA-encoding genes that is imperative for normal skeletal development and body growth in mammals. miR-214 is transcribed together with miR-199a-2 from the opposite strand of the Dnm3 gene. *In vitro*, knockdown of miR-214 activity increased osteoblastogenesis.^{35,36} Activating transcription factor 4 (ATF4) is a critical substrate of ribosomal S6 kinase 2 (RSK2) that is required for osteoblast terminal differentiation and for maintaining mature osteoblast function, including synthesis of bone sialoprotein, collagen, and osteocalcin.^{37,38} The requirement of ATF4 in osteoblast differentiation was revealed with ATF4 knockout mice. *Atf4*^{−/−} mice displayed a dramatic decrease in osteocalcin expression together with severe reduction of bone mass and bone formation.³⁸ In osteoporosis patients, miR-214 levels in bone are inversely correlated with mRNA levels for osteocalcin and alkaline phosphatase (ALP), and miR-214 was increased in ovariectomized mice.¹⁴ Importantly, knockdown of miR-214 activity in mice, using a bone-targeting antagomir, increased bone formation in normal mice, and in mice subjected to ovariectomy or hindlimb unloading.¹⁴ *In vitro* studies indicated that miR-214 targets ATF4, a transcription factor important for osteocalcin expression, osteoblastic differentiation, and amino acid import.³⁹ In addition, ATF4 promoted angiogenesis by increasing the expression and release of vascular endothelial growth factor (VEGF); and angiogenesis is critical for bone repair.⁴⁰ In addition, ATF4 induced the expression of Indian hedgehog (Ihh) in chondrocytes; Ihh is a paracrine osteogenic factor.⁴¹

Thus, delivering miR-214 anti-sense oligonucleotides (AS-miR-214) may facilitate repair mediated by endochondral or intramembranous ossification.

Previously, we demonstrated the feasibility of generating silk-based orthopedic devices using an all-aqueous process.² The uniquely mild processing conditions allow for the combination of bioactive molecules, including drugs, growth factors, and small RNAs into or post process, to functionalize the devices. The objective of this study was to optimize silk functionality for fine-tuned osteogenic outcomes by incorporating miR-214 inhibitors into the surface of silk materials.

Materials and Methods

Preparation of aqueous silk solution

Silk fibroin solution was prepared from *Bombyx mori* cocoons using our established protocols with some modifications.³² First, sericin was removed by boiling the cocoon pieces in 0.02 M aqueous Na₂CO₃ solution for 30 min followed by extensive rinses in distilled water. The degummed silk was then dried overnight and dissolved in 9.3 M LiBr at 60°C for 4 h, yielding a 20% (w/v) solution. The pH of LiBr solution was adjusted by adding 1 M LiOH solution so that the pH of the final silk solution after dialysis was 8.0. The silk/LiBr solution was dialyzed against distilled water for 2 days with 10 changes of water. The solution was then centrifuged for 2 × 20 min at 9000 rpm. The silk concentration was determined by evaporating water from a solution of known weight and weighing the remaining solid using an analytical balance.

Preparation of silk films

Briefly, 7% w/v silk solution was cast onto 24-well dishes. The silk films were dried at room temperature overnight and water annealed with 150 nM concentrations of AS-miR-214 strands (AS-miR-214 silk films), nontargeting AS strand controls (Ctrl AS-miR Silk Films) (Dharmacon, Lafayette, CO), or silk films alone, Table 1. Before adding miRNAs to the silk, inhibitors were mixed with Xfect RNA Transfection Reagent (Clontech, Mountain View, CA), as per the manufacturer's instructions.

Machining of silk orthopedic devices

The silk screws were developed as previously described.² Briefly, silk solution of 7–9% (wt/wt) was subjected to forced airflow and water was slowly removed at 10°C until the silk concentration reached 25–30%. The concentration of the solution was determined by weighing the remaining material after drying. Rectangular molds with water-permeable membranes were used to prepare silk blanks for machining. Silk screw blank molds were prepared using wax from MachinableWax.com and machined to 1.3 in (3.30 cm) height with six through holes of 0.30 in (0.76 cm) diameter. A thin wafer of 0.10 in (0.25 cm) was also machined to act

TABLE 1. LIST OF SILK BIOMATERIALS USED

Sample code	miRNA sequence content	Bioactivity
Silk films	None	None
Ctrl AS-miR silk films	UCACAACCUCCUAGAAAGAGUAGA	Nontargeting molecule
AS-miR-214 silk films	ACAGCAGGCACAGACAGGCAGU	Inhibits endogenous 214

as a base for the mold. The thin wafer was attached to the base of the mold by melting both edges of the wax and then allowing it to dry. Silk plate blank molds were prepared using wax that was machined into a rectangular mold with inner dimensions of $1.75 \times 1.75 \times 1.3 \text{ in}^3$ ($4.45 \times 4.45 \times 3.30 \text{ cm}^3$). Next, the molds were machined into screws using a CNC lathe (Trak TRL 1440 EX; Southwestern Industries, Rancho Dominguez, CA). For screws with bone screw threads, a custom single-point external cutter (Vargus, Janesville, WI) was used on the CNC lathe to cut screw threads by matching turning speed with horizontal speed of the cutter to cut a desired pitch length (outer diameter $\sim 1.8 \text{ mm}$, pitch $1/4 \text{ 600 mm}$). The screw heads were machined to have cylindrical heads by use of the CNC lathe. Once machined, the screws or IM nails were cut off behind the head or length of nail. A diamond cutter was mounted to the lathe and used to cut a slot in the screw head for screw insertion. For plates, rectangular silk blanks were machined using a CNC milling machine (Trak DPM; Southwestern Industries). The milling machine was used to cut the desired shape of the mold and thickness. Once the plate was shaped and sized, cylindrical or conical screw holes were cut using a 90 Ford countersink. The major diameter of the screw was $\sim 1.8 \text{ mm}$ and the pitch was $600 \mu\text{m}$.

Preparation of fluorescently labeled miRNA surface-coated silk screws

To confirm the incorporation of miRNAs within the silk devices, Dy547-labeled miRNAs were used. The Dy547-labeled scrambled miRNA (Dharmacon), Xfect complex was mixed into 7% silk solution described above, and dip coated with 7–8% liquid silk onto silk screws. The screws were then air dried and visualized (Keyence BZ-X700; Itasca, IL).

In vitro release of AS strand miR-214 from silk films

The release kinetics of miR-214 inhibitors was determined by incubating silk films with AS-miR-214 ($n=6$) in $300 \mu\text{L}$ phosphate-buffered saline (PBS) (pH 7.4) at 37°C for up to 7 days. Released miRNA inhibitor was quantified by RiboGreen Assay (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions and read using a NanoDrop spectrophotometer (Thermo Fisher Scientific). The results are reported as cumulative release in cumulative percent.

Human mesenchymal stem cell isolation and expansion

Human mesenchymal stem cell (hMSCs) isolation was from total bone marrow aspirate from a healthy, nonsmoking male under the age of 25 (Lonza, Walkersville, MD). Whole bone marrow was diluted in expansion medium, plated in 175-cm^2 flasks (Corning) at a density of $200,000 \text{ cells/cm}^2$ and cultured at 37°C with 5% CO_2 in a humidified environment. Expansion media consisted of Dulbecco's modified Eagle's medium (DMEM), 10% fetal bovine serum (FBS), 1% nonessential amino acid, 1 ng/mL beta fibroblast growth factor, and 1% pen-strep (Thermo Fisher Scientific). Each flask contained a final volume of 35 mL , which was rocked daily to allow hematopoietic cells to remain in suspension and the stromal cells to adhere to the

flasks. The adherent cells reached 80% confluence, after which they were trypsinized, suspended in FBS containing 10% DMSO, and stored in liquid nitrogen. Second passage cells were used for experiments.

Human umbilical vein endothelial cells

Human umbilical vein endothelial cells (HUVECs) (Lonza) were cultured in endothelial growth factor media (Lonza) with 1% antibiotic–antimycotic (Thermo Fisher Scientific). Cells from passage 6–10 were utilized in these experiments. The culture media were replaced every 2–3 days.

Examination of cellular viability

The relative metabolic activity of cells seeded on silk–miRNA films was determined by alamarBlue assay (Life Technologies, Grand Island, NY) according to the manufacturer's directions. The different films were cast into a 24-well Costar cell culture plates (Sigma-Aldrich, St. Louis, MO). Cells were allowed to grow on silk films for various predetermined time points (up to 3 weeks and incubated with 10% alamarBlue reagent for 4 h at 37°C). Following incubation, aliquots of $100 \mu\text{L}$ were placed into black 96-well plates and fluorescence was quantified using a spectrofluorometer with the peak excitation and emission wavelengths of 530 and 560 nm, respectively, using a Synergy HT Multi-Mode Microplate Reader (Bio-Tek Instruments, Winooski, VT). Acellular films were used to normalize background fluorescence.

Immunostaining

All samples were fixed with 4% paraformaldehyde for 15 min and then washed in PBS. Cells were incubated in 0.1% Triton-X for 15 min, followed by washing in PBS. Then 5% normal blocking serum in PBS was added to each sample for 20 min to suppress nonspecific binding of IgG. Samples were then incubated in primary antibody diluted (1:100) (R&D Systems, Minneapolis, MN) in 3% normal blocking serum for 60 min and used in accordance with the manufacturer's instructions. After PBS washing, samples were incubated for 45 min in a dark chamber with FITC-conjugated secondary antibody (Abcam, Cambridge, MA) diluted 1:200 in 1% normal blocking serum.

ALP staining

To assess early osteoinductivity, hMSCs were cultured with osteogenic medium containing 10 nM dexamethasone, 10 mM β -glycerophosphate, and $50 \mu\text{g/mL}$ ascorbic acid for 2 weeks and stained with commercially available Vector Blue (Invitrogen, Carlsbad, CA). For ALP staining, cells were fixed in 4% paraformaldehyde for 15 min at room temperature and washed with PBS. Wells were then processed for ALP staining using the ALP Vector Blue Kit (Vector Laboratories, Burlingame, CA) as per the manufacturer's instructions. After incubating cells in ALP staining solution for 10 min, cells were rinsed in deionized water twice and imaged. Fluorescence intensity was quantified at an excitation of 500 nm and emission of 680 nm using a Synergy HT Multi-Mode Microplate Reader (Bio-Tek Instruments).

Crystal violet staining

Crystal Violet assay was used to determine cell colony attachment and thus presence by binding to DNA content. hMSCs were seeded at a density of 3.5×10^4 cells/well ($n=4$) on silk films, Ctrl AS-miR silk films, or AS-miR-214 silk films. The extent of cellular attachment on the matrices was followed after ALP staining. At this time point, cells were washed three times with PBS, fixed with 10% formaldehyde, and stained with 0.5% (w/v) Crystal Violet solution for 10 min. After washing off the excess dye with water, the Crystal Violet-positive cells were solubilized with 1% trypsin solution, and absorbance was measured at 570 nm on a Synergy HT Multi-Mode Microplate Reader (Bio-Tek Instruments).

Examination of live culture mineralization

Xylenol Orange was added to the medium overnight at a concentration of 20 μ M and allowed to bind to newly formed calcium deposits. Xylenol Orange expressed a red color under a TRITC Red filter.⁴² The cell cultures were imaged using a fluorescence microscope (Keyence BZ-X700; Itasca).

Confirmation of mineralization using von Kossa

To confirm mineralization, hMSCs were cultured with the osteogenic medium containing 10 nM dexamethasone, 10 mM β -glycerophosphate, and 50 μ g/mL ascorbic acid for 3 weeks and stained using Silver Nitrate staining according to the von Kossa method. Briefly, cells were washed with PBS and fixed with 4% paraformaldehyde. The von Kossa staining was initiated by the addition of 5% Silver Nitrate solution, and exposure to UV light for 1 h. The reaction was terminated by rinsing with deionized water and neutralizing the residual Silver Nitrate with 5% sodium thiosulfate. Mineralized nodules were seen as dark brown to black spots. The cell cultures were imaged using a fluorescence microscope (Keyence BZ-X700; Itasca).

Statistical analysis

Data were statistically analyzed and expressed as mean $\pm$ standard deviation. One-way ANOVA followed by

Tukey's test or Student's *t*-test was performed. **p* values <0.05 were considered statistically significant.

Results and Discussion

Detection of surface-coated miRNAs in silk screws

Previously, we demonstrated the capacity to load silk-based orthopedic devices with the antimicrobial drug Ciprofloxacin,² the osteoinductive growth factor bone morphogenic protein 2 (BMP2),^{1,2} as well as the BMP2's peptide P24.² The study demonstrated the capacity to load different bioactive molecules, as well as the capacity for these molecules to maintain functionality. In spite of significant bioactivity and clinical use, proteins can also have adverse side effects, including immune reactions as well as ectopic and heterotopic bone formation. This has been attributed to the supra-physiological doses used clinically, which are frequently close to 1 million times greater than endogenous levels.^{43–46} Hence, the use of low concentrations of miRNAs as an alternative transient approach to stimulate long-term endogenous growth factor expression may provide an ideal alternative. Due to the relative instability of miRNAs compared with growth factors, it is advantageous to use the aqueous processed silk-based material to sequester solvent-based impairment of RNA activity. Furthermore, the negative charge of silk allows for the use of mild commercially available nonviral cationic transfection reagents, such as Lipofectamine, TKO, or Xfect to facilitate cellular uptake. The use of small amounts of nontoxic transfection reagents similar to Xfect has been shown to help facilitate and increase the transfection efficiency without affecting the development or loading concentrations in scaffolds.^{14,15} In this study, we choose to use Xfect, a proprietary biodegradable polymer chosen due to its low toxicity and high delivery efficiency in the presence of a serum. This interaction results in a net positive charge for the miRNA nanocomplex and provides a means for the nucleic acids to override the electrostatic repulsion of the negatively charged silk, allowing fusion with the surface of cells. These electrostatic interactions are useful for surface stability and delivery. Figure 1A–F shows phase

FIG. 1. Fluorescence micrographs of Dy547-tagged miRs incorporation and release kinetics. (A–C) Silk without miRs and (D–F) silk with fluorescently labeled miRs. Phase contrast image, fluorescent miRs (scale bar = 100 μ m). (G) Release kinetics of AS-miR-214 from silk films. Release reported as cumulative release of percent, up to 7 days.

contrast and fluorescence microscopy images of silk screws coated with or without Dy547-labeled miRNAs. Autofluorescence was not detected on the surface of the silk screws (Fig. 1A, C). In contrast, even fluorescence was observed from the silk devices coated with the Dy547-labeled miRNA (Fig. 1D, F). Hence, miRNA dip coating method appears robust, densely coated over the entire exterior of the screw.

In vitro release of AS strand miR-214 from silk films

After determining whether miRNAs could be incorporated on the surface of silk-based devices (screws), we investigated the release kinetics of miRNAs from the silk. Silk films were used for these studies as a representative device format to investigate surface modifications using miRNAs to assess cellular interactions. This initial 2D systems was selected for the improved control and analytics of the systems. An efficient functionalized device system should exhibit optimal concentrations of miRNA and subsequent release of this cargo. Cells are typically transfected within 24 h and have bioactivity up to 2 or 3 weeks.^{14,47} To understand how the miRNA is released from the silk, a short-term release study was performed. Figure 1G demonstrates the release kinetics of AS-miR-214 from the silk films, based on incubation in PBS at 37°C for up to 168 h. Samples of PBS were removed from each respective time point and replaced with fresh PBS. The released miRNA inhibitor was quantified by RiboGreen Assay (Thermo Fisher Scientific). The silk films showed an initial release of ~20% AS-miRNAs within the first 24 h, followed by the continued or sustained release of an additional 42% of the miRNA load in the next 48 h. The silk released an additional 36% after 168 h. In another study, chitosan nanoparticle thin films were complexed with small RNAs and surface coated as layer by layer films onto the surface of titanium to promote osteogenesis.⁴⁸ The release kinetics of siRNAs revealed a similar distribution, likely due to the similarity in molecule size (21–24 nucleotides) and the highly positive charge^{49,50} of the chitosan bound as the delivery vehicle, similar to our

Xfect reagent. The continued release of miRNAs from silk mimics the distribution of miRNAs in traditional transfection systems *in vitro*.

Viability of hMSCs on AS-miR-214 silk films

To determine whether transfection facilitated with Xfect from silk films had an adverse effect on cell viability, alamarBlue assay was performed on the hMSCs seeded on silk films. Cells were seeded on silk films loaded with AS-miR-214 or silk films alone (Fig. 2A). hMSCs seeded on silk films exhibited significantly higher cell viability ($*p < 0.05$) compared with both the scrambled negative control-loaded silk films and the AS-miR-214-loaded silk films after 72 h. On day 7, the remaining cells yielded higher metabolic activity ($*p < 0.05$) seeded on silk films alone than the scrambled negative control-loaded silk films and the AS-miR-214-loaded silk films. However, no differences were found among silk films and scrambled sequence on or after 14 days of culture. Cells seeded on AS-miR-214-loaded silk films exhibited lower metabolic activity ($*p < 0.05$) than on both the silk films alone and the silk films loaded with the scrambled sequence for the duration of the study. To determine whether AS-miR-214 silk films had an adverse effect on cellular attachment and/or apoptosis, actin filament staining was performed (Fig. 2B, C). Interestingly, cells seeded on silk films alone appeared robust and healthy after 24 h, comparable to cells transfected with AS-miR-214, with no clear difference in morphology. Moreover, no groups appeared apoptotic (Fig. 2B, C). Noteworthy, the presence of anabolic induction agents was demonstrated to slow the proliferation of MSCs, allowing for physiological cues to drive the stem cell lineage toward a terminal osteogenic lineage. Senescence is a concern related to bone formation in post irradiation (IR) treatment.^{51–54} Additionally, proliferation of both hMSCs and osteoblasts can be severely compromised after treatment of IR-induced senescence.⁵⁵ However, senescent hMSCs are delineated by their inability to form mineralized deposits and lack of ability

FIG. 2. Cell viability and morphology of hMSCs after AS-miR silk films mediated transfection. (A) alamarBlue assay for cell viability comparing all groups for 21 days. (B) Actin staining after cells were seeded on silk or AS-miR-214 silk films (C). Scale bar = 100 μ m. ($n = 3$, $*p < 0.05$) indicates hMSCs seeded on silk films and scramble-loaded silk films viability as significantly higher than hMSCs seeded on AS-miR 214 films. hMSC, human mesenchymal stem cell.

to be rescued to their previous capacity to mineralize.^{51–53,55} In the present work, the lower metabolic activity may be in part due to some cells undergoing senescence, however, the hMSCs maintained their mineralizing capacity. The presence of healthy cells with lower metabolic activity suggests that cells may be committing to the osteogenic lineage, and thus fewer cells may enter the mitotic stage.

AS-miR-214 silk film decoys transcription-induced osteogenesis

We next determined whether the decrease in metabolic activity was due to miR-214 inhibitor uptake and subsequent osteoinduction. ALP is an early marker expressed during osteoblastic differentiation.^{56–59} ALP increases the amount of inorganic phosphate while reducing the amount of extracellular pyrophosphate, which are inhibitors of phosphorylation.⁶⁰ Thus, ALP plays a vital role in osteogenesis and matrix mineralization.^{58,60} ALP expression was first evaluated by staining the hMSCs seeded on AS-miR-214 silk films, AS-scrambled negative control silk films, and silk alone at day 21 in osteogenic media (Fig. 3A–D). Cells seeded on AS-miR-214 silk films exhibited punctate positive ALP expression, indicated by the blue stain, whereas both AS-scrambled negative control silk films and silk films alone exhibited more diffuse ALP expression (Fig. 3B–D). Crystal Violet is a staining method used to bind DNA, typically performed to determine cellular attachment and cell colony detection. In this study, Crystal Violet assay was used to identify cell colonies at day 21 (Fig. 3A). The cell colonies present in both control groups were more abundant when compared with Crystal Violet-positive cells in the AS-miR-214 silk films. Normalization of ALP-positive cells to hMSCs to cell colonies demonstrated a significantly higher amount of ALP expression ($p < 0.05$) from cells seeded on the AS-miR-214 silk films, comparatively (Fig. 3D). The

results suggested that although fewer cell colonies were present among the AS-miR-214 silk film group, more cells produced the osteogenic marker ALP, confirming enhanced osteoinduction. It was previously reported that bone marrow-derived stem cell proliferation may decelerate in the presence of the induction agents.^{61–63}

AS-miR-214 silk film enhanced matrix mineralization

After confirming an enhanced early marker for an osteogenic profile by hMSCs cultured on AS-miR-214 silk films, another study was performed to investigate whether the scaffolds enhanced matrix mineralization, a later osteoblastic parameter. hMSCs were seeded on AS-miR-214 silk films or scrambled negative miR load silk films, for 14 days, and calcium content was quantified on live cells using Xylene Orange (XO) staining (Fig. 4A, B). XO is a calcium chelator, which binds to calcium deposits and fluoresces under a TRITC filter. Increased XO staining was observed in the AS-miR-214 group compared with the scrambled group and silk alone. Cells seeded on nontargeting control scramble-loaded silk deposited little calcium (Fig. 4B), whereas cells seeded on AS-miR-214-loaded films displayed robust intense XO expression (Fig. 4A). hMSCs were allowed to grow for an additional week and stained for ALP and von Kossa colocalization staining for confirmation for late-stage osteogenesis and calcium deposition (Fig. 5A, B). Increased calcium deposition was confirmed by black and brown spots observed in the AS-miR-214 group (Fig. 5B) compared with the relative absence of these spots located on the nontargeting control scramble-loaded silk films (Fig. 5A). Additionally, the enhancement of ALP activity was evident by the relatively strong expression of blue visible in the AS-miR-214-loaded films compared with the scramble controls. Overall, the data provide a rationale for further validating the system as a potent, transient delivery

FIG. 3. ALP expression of hMSCs after 14 days seeded on AS-miR-214 silk films mediated transfection. (A) Crystal Violet staining (DNA content) and ALP expression. hMSCs seeded on (B) control AS-miR ALP expression and (C) AS-miR-214 silk films. (D) Quantification of ALP expression normalized to DNA content ($n = 3$, $*p < 0.05$) (scale bar = 100 μ m. ALP, alkaline phosphatase).

FIG. 4. Xylene Orange staining for calcium deposition of living hMSCs cultured on AS-miR-214 silk films mediated transfection after 14 days. (A) hMSCs on control AS-miR, (B) AS-miR-214 silk films, and (C) quantification of Xylene Orange staining normalized to DNA content ($n = 5$, $*p < 0.05$).

vehicle with bone regenerative potential. It is likely that the enhanced calcium deposition was due to AS-miR-214 acting as a decoy to suppress endogenous transcripts to allow for upregulation of the ATF4 expression as discussed earlier.

HUVECs-hMSCs coupling on AS-miR-214 silk films enhances early osteoinductivity

The intricate relationship between endothelial cells (ECs) and hMSCs *in vivo* must be replicated during the bone repair process to ensure the formation of a functional vascular network to support successful bone formation. Crosstalk between hMSCs and ECs is recognized as one of the most important cellular interactions crucial for maintenance and

bone formation.^{64,65} A recent study developed a global map of gene expression following coculture of MSCs with ECs for extended times in a direct-contact *in vitro* system.⁶⁵ The findings demonstrated that coculturing of the two cell types upregulated genes related to angiogenesis, such as VEGF and platelet-derived growth factor, as well as genes, which play a crucial role in osteogenesis, such as ALP and BMP2. These findings demonstrated that coculturing may lead to enhanced regulation of osteogenesis. Interestingly, miR-214 overexpression reduced angiogenesis and proliferation of HUVECs by targeting x-box-binding protein 1 (XBP1), a crucial factor of unfolded protein response. Furthermore, miR-214 also directly targets VEGF, a vital growth factor for bone formation. Hence, coupling endothelial-hMSCs while inhibiting miR-214 could result in synergistic promotion of osteoinductivity. Figure 6A–P, shows how HUVECs and hMSCs were cocultured on AS-miR-214 silk films, and silk films with negative control scramble in osteogenic media and stained for the EC marker, target molecule ATF4, and osteogenic marker Runx2 expression. Endothelial marker CD31 expression (Fig. 6A and E), appeared to be robust and expressed at similar levels among ECs seeded on both AS-miR-214 silk films and the control group. The study also demonstrated colocalization of ATF4 with the expression and upregulation of the early osteogenic master regulator Runx2 in cells seeded on AS-miR-214 silk films compared with cells seeded on control (scrambled) silk films (Fig. 6C, G).

Additionally, we also identified upregulation of the ATF4 downstream target molecule and key osteogenic regulator osteocalcin (Fig. 6K, O). In addition, there was a significant increase in ALP expression, in cultures on the AS-miR-214 silk films compared with cells seeded on scramble-loaded silk films (Fig. 6I, M). The cultures exhibited punctate dark blue staining similar to hMSCs cultured alone. Hence, the coupling of ECs with hMSCs had the capacity to promote osteogenesis in a complimentary manner. The combination of osteogenic marker protein expression, ALP expression, and mineralization of hMSCs and HUVECs-hMSC cocultures on miR-loaded silk films established the capacity for surface-modified miRNA silk-based devices to control cell fate and function.^{66–68} Overall, these data provide a rationale for further validating the system as a potent, transient delivery vehicle with bone regenerative potential.

AS-miR-214 silk screw-mediated transfection enhances osteogenesis and mineralization

Currently, many orthopedic devices function in a suitable manner mechanically, but are insufficient for osseointegration

FIG. 5. ALP and von Kossa staining for osteoinductivity and mineralization of hMSC-cultured AS-miR-214 silk films mediated transfection after 21 days. (A) hMSCs on control AS-miR, and (B) AS-miR-214 silk films. Black indicates mineral, (scale bar = 100 μ m ($n = 5$)).

FIG. 6. Colocalization of target molecule ATF4, Runx2, and CD31 expression in HUVEC/hMSCs cocultured AS-miR-214 silk films mediated transfection after 14 days. (A) Target molecule ATF4 expression, (B) CD31, (C) Runx2, (D) merge of cocultures seeded on AS-miR control; (E) ATF4 and (F) CD31 (G) Runx2 (H) merge of cocultures seeded on AS-miR-214 silk films. Additionally, (I) ALP expression, (J) CD31, (K) osteocalcin, (L) merge of cocultures seeded on AS-miR control; whereas (M) ALP expression, (N) CD31, (O) OCN, and (P) merge of cocultures seeded on AS-miR-214 silk films (representative image, $n=5$ scale bar = 300 μm). ATF4, activating transcription factor 4; HUVECs, human umbilical vein endothelial cells.

and vascularization with the surrounding bone formation. This leads to undesirable implant migration, loosening, and ultimately mechanical destabilization. Hence, next we investigated the capacity for AS-miR-214 surface-coated silk-based screw capacity to enhance osseointegration and angiogenesis as a means to mitigate adverse events associated with the lack of integration exhibited by traditional devices. Figure 7A–H, shows eGFP⁺-expressing HUVECs and nontagged hMSCs were cocultured on AS-miR-214 silk screws, and silk screw with scramble in osteogenic media for 14 days. The cocultures were then fixed and stained for the late-stage mature bone marker osteocalcin (Fig. 7B, F). AS-miR silk screws with nontargeting microRNAs exhibited little-to-no osteocalcin expression (Fig. 7B), whereas, AS-miR-214 silk screws exhibited robust osteocalcin expression from cells throughout the structure of the screw (Fig. 7F). Both screws exhibited eGFP⁺ expression of HUVECs (Fig. 7D, H); however, eGFP⁺ HUVECs cultured on the scramble-loaded control screw appeared to be mainly on the threads of the screw only (Fig. 7D).

Conversely, HUVECs located on the AS-miR-214 silk screw demonstrated eGFP⁺ HUVECs located on the body, threads, and head of the screw, demonstrating an organized biomimetic promotion of vascular network formation. This suggests that cells that were not in the presence of AS-miR-214 were heavily influenced by surface topography, whereas cells seeded on AS-miR-214 screws were guided by targeted protein modulation. These results suggest that not only does AS-miR-214 screws exhibit an osteoinductive niche, but these screws exhibit a bidirectional synergistic environment, by simultaneously promoting organization of angiogenic networks.

In addition, to protein expression we examined ALP expression. Expression of ALP from cells seeded on scramble-loaded silk screws exhibited limited and random positive expression throughout the screw indicated by positive dark blue staining (Fig. 7L). On the other hand, cells seeded on AS-miR-214 silk screws exhibited robust positive expression on the body of the screw located above each thread, including the head of the screw in a more organized fashion

FIG. 7. AS-miR-214 silk screw transfection mediated osteoinduction and mineralization in HUVEC/hMSCs cocultured after 14 days. (A) DAPI, (B) osteocalcin, (C) merge, and (D) eGFP⁺ HUVECs with hMSCs cocultures seeded on AS-miR control silk screws; (E) DAPI, (F) osteocalcin, (G) merge, and (H) eGFP⁺ HUVECs with hMSCs cocultures seeded on AS-miR-214 silk screws; (I-K) von Kossa staining with silk autofluorescence of cocultures seeded on AS-miR control silk screws; (L) ALP expression of cocultures on control AS-miR silk screws and AS-miR-214 silk screws. (M-O) von Kossa staining with silk auto fluorescence of cocultures seeded on AS-miR-214 silk screws, (P) quantification of von Kossa staining ($n=5$, $*p<0.05$) (representative image, $n=3$, scale bar = 300 μ m).

(Fig. 7L). Similarly, von Kossa staining revealed a similar profile of calcium deposition of scramble-loaded silk screws confirmed by randomly located black staining (Fig. 7I, K). In contrast, von Kossa staining on the AS-miR-214 screws demonstrated a significantly higher calcium deposition on the majority of the screw surface, body, and head, comparatively as seen in Fig. 7M, O, and P. These results demonstrated that a microRNA milieu mediated by silk-based biomaterials could be a unique platform to ensure more robust and suitable orthopedic devices for bone fracture fixation and promotion of enhanced healing.

Conclusions

In summary, we demonstrated the feasibility of developing an all-aqueous silk-based device for miRNA-based therapeutics, aimed at enhancing osteoblastic commitment and osseous integration. Incorporation of miRNAs onto the surface of silk-based screws exhibited a smooth and uniform coating. Additionally, miRNAs exhibited a burst release profile from silk materials, followed by continuous release for up to 7 days. In addition, we demonstrated good cytocompatibility with no apparent adverse effects. Moreover, AS-miR-214-loaded silk films and screws both significantly enhanced osteogenic differentiation markers and mineralization, providing a means to control cellular differentiation and function of both osteogenesis and angiogenesis in a synergistic approach. Collectively, this study demonstrated the feasibility of producing silk-based devices to facilitate enhanced osteogenesis. The unique characteristics of aqueous silk-based devices, combined with their ability to present miRNA-based therapeutics in a transient, bioactive manner with extended activity, may serve as a novel method of next-generation bone tissue repair and regeneration.

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Disclosure Statement

No competing financial interests exist.

References

1. Koolen, P.G.L., Haas, D., Kim, K., *et al.* Increased osteoid formation in BMP-2-loaded silk-based screws. *Plastic Reconstr Surg* **137**, 808e, 2016.
2. Li, C., Hotz, B., Ling, S., *et al.* Regenerated silk materials for functionalized silk orthopedic devices by mimicking natural processing. *Biomaterials* **110**, 24, 2016.
3. Maillard, Y.P. Fine-structure of surface of screw plate and cocoon silk of egg-laying of hydrophilidae (Insecta, Coleoptera). *Comptes Rendus* **275**, 75-8, 1972.
4. Perrone, G.S., Leisk, G.G., Lo, T.J., *et al.* The use of silk-based devices for fracture fixation. *Nat Commun* **5**, 3385, 2014.
5. Altman, G.H., Diaz, F., Jakuba, C., *et al.* Silk-based biomaterials. *Biomaterials* **24**, 401, 2003.
6. Sofia, S., McCarthy, M.B., Gronowicz, G., and Kaplan, D.L. Functionalized silk-based biomaterials for bone formation. *J Biomed Mater Res* **54**, 139, 2001.
7. Zhao, X., Xu, D., Li, Y., *et al.* MicroRNAs regulate bone metabolism. *J Bone Miner Metab* **32**, 221, 2014.
8. Papaioannou, G., Mirzamohammadi, F., and Kobayashi, T. MicroRNAs involved in bone formation. *Cell Mol Life Sci* **71**, 4747, 2014.
9. Moore, B.T., and Xiao, P. MiRNAs in bone diseases. *Microna* **2**, 20, 2013.
10. Inose, H., Ochi, H., Kimura, A., *et al.* A microRNA regulatory mechanism of osteoblast differentiation. *Proc Natl Acad Sci U S A* **106**, 20794, 2009.
11. Ell, B., and Kang, Y. MicroRNAs as regulators of bone homeostasis and bone metastasis. *Bonekey Rep* **3**, 549, 2014.
12. Kapinas, K., and Delany, A.M. MicroRNA biogenesis and regulation of bone remodeling. *Arthritis Res Ther* **13**, 220, 2011.
13. Burnett, J.C., and Rossi, J.J. RNA-based therapeutics: current progress and future prospects. *Chem Biol* **19**, 60, 2012.
14. Wang, X., Guo, B., Li, Q., *et al.* miR-214 targets ATF4 to inhibit bone formation. *Nat Med* **19**, 93, 2013.
15. James, E.N., Delany, A.M., and Nair, L.S. Post-transcriptional regulation in osteoblasts using localized delivery of miR-29a inhibitor from nanofibers to enhance extracellular matrix deposition. *Acta Biomater* **10**, 3571, 2014.
16. Pfeifer, A., and Verma, I.M. Gene therapy: promises and problems. *Annu Rev Genomics Hum Genet* **2**, 177, 2001.
17. Hu, R., Li, H., Liu, W., Yang, L., Tan, Y.F., and Luo, X.H. Targeting miRNAs in osteoblast differentiation and bone formation. *Expert Opin Ther Targets* **14**, 1109, 2010.
18. Jacques, C., Calleja, L.R., Baud'huin, M., *et al.* miRNA-193a-5p repression of p73 controls Cisplatin chemoresistance in primary bone tumors. *Oncotarget* **7**, 54503, 2016.
19. Paquet, J., Moya, A., Bensidhoum, M., and Petite, H. Engineered cell-free scaffold with two-stage delivery of miRNA-26a for bone repair. *Ann Transl Med* **4**, 204, 2016.
20. Lee, W.Y., Li, N., Lin, S., Wang, B., Lan, H.Y., and Li, G. miRNA-29b improves bone healing in mouse fracture model. *Mol Cell Endocrinol* **430**, 97, 2016.
21. Zhang, X., Li, Y., Chen, Y.E., Chen, J., and Ma, P.X. Cell-free 3D scaffold with two-stage delivery of miRNA-26a to regenerate critical-sized bone defects. *Nat Commun* **7**, 10376, 2016.
22. Shen, X., Guo, Y., Yu, J., *et al.* miRNA-202 in bone marrow stromal cells affects the growth and adhesion of multiple myeloma cells by regulating B cell-activating factor. *Clin Exp Med* **16**, 307, 2016.
23. Bae, Y., Yang, T., Zeng, H.C., *et al.* miRNA-34c regulates Notch signaling during bone development. *Hum Mol Genet* **21**, 2991, 2012.
24. Zhang, X.H., Geng, G.L., Su, B., Liang, C.P., Wang, F., and Bao, J.C. MicroRNA-338-3p inhibits glucocorticoid-induced osteoclast formation through RANKL targeting. *Genet Mol Res* **15**, 2016.
25. Sun, W., Zhao, C., Li, Y., *et al.* Osteoclast-derived microRNA-containing exosomes selectively inhibit osteoblast activity. *Cell Discov* **2**, 16015, 2016.
26. Kim, K., Kim, J.H., Kim, I., *et al.* MicroRNA-26a regulates RANKL-induced osteoclast formation. *Mol Cells* **38**, 75, 2015.
27. Sun, K.T., Chen, M.Y., Tu, M.G., Wang, I.K., Chang, S.S., and Li, C.Y. MicroRNA-20a regulates autophagy related protein-ATG16L1 in hypoxia-induced osteoclast differentiation. *Bone* **73**, 145, 2015.
28. Shi, C., Qi, J., Huang, P., *et al.* MicroRNA-17/20a inhibits glucocorticoid-induced osteoclast differentiation and function through targeting RANKL expression in osteoblast cells. *Bone* **68**, 67, 2014.

29. Lee, Y., Kim, H.J., Park, C.K., *et al.* MicroRNA-124 regulates osteoclast differentiation. *Bone* **56**, 383, 2013.
30. Shibuya, H., Nakasa, T., Adachi, N., *et al.* Overexpression of microRNA-223 in rheumatoid arthritis synovium controls osteoclast differentiation. *Mod Rheumatol* **23**, 674, 2013.
31. Sugatani, T., and Hruska, K.A. MicroRNA-223 is a key factor in osteoclast differentiation. *J Cell Biochem* **101**, 996, 2007.
32. Kalyani, A., Sonawane, P.J., Khan, A.A., *et al.* Post-transcriptional regulation of renase gene by miR-29 and miR-146 microRNAs: implications for cardiometabolic disorders. *J Mol Biol* **427**, 2629, 2015.
33. Li, Y., Fan, L., Liu, S., *et al.* The promotion of bone regeneration through positive regulation of angiogenic-osteogenic coupling using microRNA-26a. *Biomaterials* **34**, 5048, 2013.
34. Li, Y., Fan, L., Hu, J., *et al.* MiR-26a rescues bone regeneration deficiency of mesenchymal stem cells derived from osteoporotic mice. *Mol Ther* **23**, 1349, 2015.
35. Watanabe, T., Sato, T., Amano, T., *et al.* Dnm3os, a non-coding RNA, is required for normal growth and skeletal development in mice. *Dev Dyn* **237**, 3738, 2008.
36. Liu, J., Li, D., Dang, L., *et al.* Osteoclastic miR-214 targets TRAF3 to contribute to osteolytic bone metastasis of breast cancer. *Sci Rep* **7**, 40487, 2017.
37. Yang, X., and Karsenty, G. ATF4, the osteoblast accumulation of which is determined post-translationally, can induce osteoblast-specific gene expression in non-osteoblastic cells. *J Biol Chem* **279**, 47109, 2004.
38. Yang, X., Matsuda, K., Bialek, P., *et al.* ATF4 is a substrate of RSK2 and an essential regulator of osteoblast biology; implication for Coffin-Lowry Syndrome. *Cell* **117**, 387, 2004.
39. Long, F. Building strong bones: molecular regulation of the osteoblast lineage. *Nat Rev Mol Cell Biol* **13**, 27, 2012.
40. Zhu, K., Jiao, H., Li, S., *et al.* ATF4 promotes bone angiogenesis by increasing VEGF expression and release in the bone environment. *J Bone Miner Res* **28**, 1870, 2013.
41. Wang, W., Lian, N., Ma, Y., *et al.* Chondrocytic Atf4 regulates osteoblast differentiation and function via Ihh. *Development* **139**, 601, 2012.
42. Wang, Y.H., Liu, Y., Maye, P., and Rowe, D.W. Examination of mineralized nodule formation in living osteoblastic cultures using fluorescent dyes. *Biotechnol Prog* **22**, 1697, 2006.
43. Boyne, P.J., Marx, R.E., Nevins, M., *et al.* A feasibility study evaluating rhBMP-2/absorbable collagen sponge for maxillary sinus floor augmentation. *Int J Periodontics Restorative Dent* **17**, 11, 1997.
44. Dahabreh, Z., Calori, G.M., Kanakaris, N.K., Nikolaou, V.S., and Giannoudis, P.V. A cost analysis of treatment of tibial fracture nonunion by bone grafting or bone morphogenetic protein-7. *Int Orthop* **33**, 1407, 2009.
45. Dahabreh, Z., Dimitriou, R., and Giannoudis, P.V. Health economics: a cost analysis of treatment of persistent fracture non-unions using bone morphogenetic protein-7. *Injury* **38**, 371, 2007.
46. Geesink, R.G., Hoefnagels, N.H., and Bulstra, S.K. Osteogenic activity of OP-1 bone morphogenetic protein (BMP-7) in a human fibular defect. *J Bone Joint Surg Br* **81**, 710, 1999.
47. Guo, Y., Li, L., Gao, J., Chen, X., and Sang, Q. miR-214 suppresses the osteogenic differentiation of bone marrow-derived mesenchymal stem cells and these effects are mediated through the inhibition of the JNK and p38 pathways. *Int J Mol Med* **39**, 71, 2017.
48. Song, W., Song, X., Yang, C., *et al.* Chitosan/siRNA functionalized titanium surface via a layer-by-layer approach for in vitro sustained gene silencing and osteogenic promotion. *Int J Nanomedicine* **10**, 2335, 2015.
49. Sadreddini, S., Safaralizadeh, R., Baradaran, B., *et al.* Chitosan nanoparticles as a dual drug/siRNA delivery system for treatment of colorectal cancer. *Immunol Lett* **181**, 79, 2017.
50. Salehi, E., Daraei, P., and Arabi Shamsabadi, A. A review on chitosan-based adsorptive membranes. *Carbohydr Polym* **152**, 419, 2016.
51. Bajada, S., Marshall, M.J., Wright, K.T., Richardson, J.B., and Johnson, W.E. Decreased osteogenesis, increased cell senescence and elevated Dickkopf-1 secretion in human fracture non union stromal cells. *Bone* **45**, 726, 2009.
52. Meng, A., Wang, Y., Van Zant, G., and Zhou, D. Ionizing radiation and busulfan induce premature senescence in murine bone marrow hematopoietic cells. *Cancer Res* **63**, 5414, 2003.
53. Ma, J., Shi, M., Li, J., *et al.* Senescence-unrelated impediment of osteogenesis from Flk1+ bone marrow mesenchymal stem cells induced by total body irradiation and its contribution to long-term bone and hematopoietic injury. *Haematologica* **92**, 889, 2007.
54. Wang, Y., Schulte, B.A., LaRue, A.C., Ogawa, M., and Zhou, D. Total body irradiation selectively induces murine hematopoietic stem cell senescence. *Blood* **107**, 358, 2006.
55. Despars, G., Carbonneau, C.L., Bardeau, P., Coutu, D.L., and Beausejour, C.M. Loss of the osteogenic differentiation potential during senescence is limited to bone progenitor cells and is dependent on p53. *PLoS One* **8**, e73206, 2013.
56. Caverzasio, J., and Manen, D. Essential role of Wnt3a-mediated activation of mitogen-activated protein kinase p38 for the stimulation of alkaline phosphatase activity and matrix mineralization in C3H10T1/2 mesenchymal cells. *Endocrinology* **148**, 5323, 2007.
57. Orimo, H. The mechanism of mineralization and the role of alkaline phosphatase in health and disease. *J Nippon Med Sch* **77**, 4, 2010.
58. Golub, E.E., Harrison, G., Taylor, A.G., Camper, S., and Shapiro, I.M. The role of alkaline phosphatase in cartilage mineralization. *Bone Miner* **17**, 273, 1992.
59. Orimo, H., and Shimada, T. The role of tissue-nonspecific alkaline phosphatase in the phosphate-induced activation of alkaline phosphatase and mineralization in SaOS-2 human osteoblast-like cells. *Mol Cell Biochem* **315**, 51, 2008.
60. Golub, E.E., Boesze-Battaglia, K., and Ellis, E. The role of alkaline phosphatase in mineralization. *Curr Opin Orthop* **18**, 444, 2007.
61. Wang, X., Ye, K., Li, Z., Yan, C., and Ding, J. Adhesion, proliferation, and differentiation of mesenchymal stem cells on RGD nanopatterns of varied nanopacings. *Organogenesis* **9**, 280, 2013.
62. Jakoby, M., and Schnittger, A. Cell cycle and differentiation. *Curr Opin Plant Biol* **7**, 661, 2004.

63. Walsh, K., and Perlman, H. Cell cycle exit upon myogenic differentiation. *Curr Opin Genet Dev* **7**, 597, 1997.
64. Kaigler, D., Krebsbach, P.H., West, E.R., Horger, K., Huang, Y.C., and Mooney, D.J. Endothelial cell modulation of bone marrow stromal cell osteogenic potential. *FASEB J* **19**, 665, 2005.
65. Xue, Y., Xing, Z., Bolstad, A.I., Van Dyke, T.E., and Mustafa, K. Co-culture of human bone marrow stromal cells with endothelial cells alters gene expression profiles. *Int J Artif Organs* **36**, 650, 2013.
66. Ko, J.Y., Chuang, P.C., Chen, M.W., *et al.* MicroRNA-29a ameliorates glucocorticoid-induced suppression of osteoblast differentiation by regulating beta-catenin acetylation. *Bone* **57**, 468, 2013.
67. Roberto, V.P., Tiago, D.M., Silva, I.A., and Cancela, M.L. MiR-29a is an enhancer of mineral deposition in bone-derived systems. *Arch Biochem Biophys* **564**, 173, 2014.
68. Kapinas, K., Kessler, C., Ricks, T., Gronowicz, G., and Delany, A.M. miR-29 modulates Wnt signaling in human osteoblasts through a positive feedback loop. *J Biol Chem* **285**, 25221, 2010.

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