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Delivery of RNAi-Based Therapeutics for Bone Regeneration

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Abstract

Purpose of review: The clinical significance, target pathways, recent successes, and challenges that preclude translation of RNAi bone regenerative approaches are overviewed.

Recent Findings: RNA interference (RNAi) is a promising new therapeutic approach for bone regeneration by stimulating or inhibiting critical signaling pathways. However, RNAi suffers from significant delivery challenges. These challenges include avoiding nuclease degradation, achieving bone tissue targeting, and reaching the cytoplasm for mRNA inhibition. Many drug delivery systems have overcome stability and intracellular localization challenges but suffer from protein adsorption that results in clearance of up to 99% of injected dosages, thus severely limiting drug delivery efficacy.

Summary: While RNAi has myriad promising attributes for use in bone regenerative applications, delivery challenges continue to plague translation. Thus, a focus on drug delivery system development is critical to provide greater delivery efficiency and bone targeting to reap the promise of RNAi.

Keywords

RNAi; siRNA; miRNA; bone regeneration; drug delivery systems

Introduction

Due to disease co-morbidities, aging, and severity of injuries, 5-10% of the ~8 million annual fractures in the United States will not fully heal. Furthermore, osteopenia and osteoporosis together afflict approximately 60 million Americans and directly contribute to

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dramatically increased risk of fragility fractures. Underlying dysfunction in cell populations, namely osteochondral progenitors (e.g., mesenchymal stem cells (MSCs)) and osteoclasts contribute to poor bone regeneration. RNA interference (RNAi) mediates post-transcriptional gene regulation [1]. The ability to silence any gene with a known sequence gives RNAi broad applicability. Small interfering RNA (siRNA) and microRNA (miRNA) are two molecules that facilitate RNAi, and recent studies have shown promising effects on bone regeneration by targeting negative regulators of regeneration [2]. siRNA delivery system development has recently enabled the first FDA-approved siRNA therapy, Patisiran, which is formulated in a lipid nanoparticle and treats a liver disease known as hereditary transthyretin amyloidosis [3]. However, significant delivery challenges still exist for siRNA. While several recent reviews overview developments of local siRNA delivery in orthopaedic applications and beyond [4-8], we focus here on systemic delivery challenges, including protein adsorption and clearance by the mononuclear phagocytic system and tissue-specific delivery. Overcoming these challenges is necessary for siRNA and/or miRNA systemic delivery to have a profound impact on non-invasive treatment for bone regeneration.

Bone Regeneration: Opportunities for siRNA Delivery

Bone regeneration is a pervasive clinical challenge. The two most common clinical indications for regenerative interventions are trauma that results in fractures or critical sized bone defects and bone loss due to osteopenia or osteoporosis. Although fractures generally heal, 5-10% of the ~8 million skeletal fractures that occur each year in the U.S. have impairments including delayed union or nonunion [9]. Impaired fracture healing, which requires prolonged or repeated treatments, has a marked impact on both patient quality of life and total healthcare costs, which tops $32 billion annually in the U.S. [10]. Osteopenia and osteoporosis together afflict approximately 60 million Americans, directly contribute to increased risk of fragility fractures [10], and result in cumulative costs estimated at $474 billion [11, 12].

Multiple cell types and growth factors are involved in successful bone regeneration [13-16]. Figure 1 depicts the cells and signals involved in endochondral ossification, which is the typical mechanism for regeneration of the appendicular skeleton. Hematoma combined with inflammation are the immediate reactions to injury, with soft tissue damage, disruption of vessels, and bone necrosis occurring simultaneously. Hematoma and inflammation initiate the release of cytokines such as stromal cell derived factor 1 (SDF1), interleukins (IL), tumor necrosis factor alpha 1 (TNFα1), platelet derived growth factor (PDGF), fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), receptor activator of nuclear factor kappa-B ligand (RANKL), and angiopoietin 1 (Ang1) to induce cell migration, proliferation, and development of new blood vessels via endothelial cells [17]. Recruited monocytes are stimulated to form osteoclasts via macrophage-colony stimulating factor (M-CSF), RANK, and RANKL to resorb bone. Transforming growth factor beta (TGFβ) and bone morphogenetic protein (BMP) are released from the bone matrix as it is remodeled by osteoclasts, and the combination induces recruited MSCs to differentiate to chondrocytes and form a soft cartilaginous callus. As healing proceeds, cartilage becomes hypertrophic and is slowly mineralized to form a hard callus and then immature woven bone. At the final remodeling stage, excess bone callus is resorbed by osteoclasts, and cycles of

osteoblast and osteoclast activity ensue, ultimately restoring bone morphology and mechanical properties.

For the axial skeleton, healing generally occurs via intramembranous ossification. After injury, Wingless-Related Integration Site (Wnt) signaling induces local MSC proliferation, osteogenesis, and condensation followed by runt-related transcription factor 2 (Runx2)-mediated production of collagen I (Col1a1) [18]. Through the effects of parathyroid hormone (PTH), BMP, and Wnt signaling, the matrix is thickened and mineralized by osteoblasts. Simultaneous to the development of intramembranous bone is angiogenesis, which requires VEGF, Ang1, FGF, and PDGF signaling. Vascular proximity provides osteoclast progenitors access to bone, where they resorb and reshape the matrix to enable final restoration of bone form and function [19].

Normal bone homeostasis, which is critical to avoid osteopetrosis or osteoporosis, is maintained through coupled formation and resorption, which is coordinated through myriad matrix-associated and soluble cues, as detailed in Figure 2 [20]. Bone-dwelling osteocytes monitor bone health and sense microcracks, which initiate osteoclast recruitment via osteocyte apoptosis and stimulates local non-apoptotic osteocyte RANKL production [21]. Osteocyte RANKL can also be upregulated through PTH stimulation, initiating bone coupling [20, 22, 23]. Similar to bone healing, RANKL recruits blood-derived monocytes, which differentiate into osteoclasts. Osteoclast-mediated release of matrix-embedded signaling molecules including TGFβ [24-27], BMP [28, 29], and IGF [30-32] then orchestrates the recruitment of osteoprogenitors to deposit new bone matrix.

.Understanding dysfunctions in the normal bone healing cascade that result in poor healing or decoupling of bone metabolism highlight potential avenues for therapeutic intervention. The cause of non-unions and delayed healing of fractures is usually unknown; however, patient-specific risk factors include age, diabetes, nicotine or alcohol use, nutritional deficiencies, infections, significant soft tissue injury, as well as chronic use of anti-inflammatories [33]. To circumvent challenges with healing, a few approaches have been approved to treat delayed healing based on pathways critical for bone regeneration (Figure 3). For example, BMP2 and 7 are approved for select non-union/delayed union interventions, while PTH or PTH related peptide (PTHrP) is used to treat osteoporosis or low bone mass and also off-label to improve nonunion or delayed healing. Additionally, therapeutic approaches that involve negative regulators of the Wnt pathway, including antibodies against dickkopf-related protein 1 (DKK1) and sclerostin, have recently been approved for osteoporosis and low bone mass treatments and may prove useful for treating nonunions or delayed unions. Despite promising clinical outcomes, the use of growth factors and antibodies is limited by their pleiotropic nature and the need for delivery of supraphysiological concentrations, which leads to side effects and significant costs [34, 35] and motivates development of alternative approaches, including RNAi technology.

Promise of siRNA

There is clear and immense potential of siRNA gene silencing techniques to promote tissue regeneration. Unlike most current small-molecule drug strategies, siRNA allows sequence-

specific inhibition of gene translation through ubiquitous native regulatory mechanisms. The specificity of inhibition is driven through complementary base pairing between the siRNA and target messenger RNA leading to the subsequent recruitment of the RNA-induced silencing complex (RISC) proteins and degradation of the target mRNA. siRNA allows the targeting of otherwise “undruggable” proteins and a greater degree of control in influencing cell behavior than existing drug treatments [2, 36].

RNAi targets for bone regeneration

siRNA

siRNA allow specific inhibition of proteins that control the differentiation, proliferation, and activity of cells involved in bone regeneration and remodeling. Cell expression of ligands, surface receptors, transcription factors, and regulatory proteins can all be modified through siRNA either by direct inhibition or inhibition of a negative regulator to increase expression of a positive regulator of remodeling/regeneration (see Figure 3). Considering this very broad range of targetable genes, insight from global gene knockout mouse models has been valuable for identifying therapeutic targets for siRNA-based approaches for bone regeneration. siRNA can be used to therapeutically emulate these gene knockouts transiently and, therefore, are amenable to translation. This approach has yielded RNAi targets in several traditionally targeted osteogenic pathways such as RANK, BMP, and Wnt signaling, but also previously undruggable targets. Table 1 summarizes reported RNAi strategies for bone regeneration.

Enzymes involved in the ubiquitination pathway have proven to be potent negative regulators of musculoskeletal regeneration. MSCs from mice deficient in several members of the Nedd4 sub-class of E3 ubiquitin ligase, including Smad ubiquitination regulatory factor (Smurf1) [37, 38], WW domain-containing E3 ubiquitin protein ligase 1 (Wwp1) [39, 40], and Itch [41], have increased cell migration, proliferation, and osteogenic differentiation. These ligases negatively regulate critical osteogenic genes including Runx2 [37] and JunB [38], and the MSC migration regulator CXC chemokine receptor 4 (CXCR4) [42]. MSCs from Itch−/−, Wwp1−/−, or Smurf1−/− mice exhibit increased osteoblast differentiation and decreased adipogenesis [37, 39-41, 43-45]. Delivery of siRNA targeting Wwp1, a negative regulator of Runx2, increased osteogenesis in mouse MSC *in vitro*. Fracture healing was expedited when delivery of siWWP1 was localized to fractures using a degradable hydrogel depot, which controlled the release of a NP delivery system for the siRNA [46]. Other work showed siRNA against noggin, a negative regulator of BMP2 signaling, in combination with miR-20, which negatively regulates adipogenesis, promotes MSC osteogenic differentiation within hydrogels [47, 48]. Additionally, delivery of siRNA against DKK1 [49] or guanidine nucleotide binding protein alpha stimulating activity polypeptide 1 (GNAS1) [50] resulted in enhanced osteogenic differentiation of MSCs.

miRNA

In contrast to siRNA, miRNA can dramatically impact the regulation of cellular programs, as a single miRNA typically has many gene targets and leads to concerted changes in protein production unlike the highly specific action of siRNA. miRNA can promote stem cell-

mediated tissue regeneration by silencing the inhibitors of differentiation-associated transcription factors. For example, miR-2861 inhibits histone deacetylase 5 (Hdac5), which is a negative regulator of the primary osteogenic transcription factor Runx2, and enhances osteogenic differentiation of mouse osteoprogenitors [51]. Additionally, delivery of a miR-21 mimic to MSCs increased osteogenesis in vitro and increased bone formation in vivo by targeting sprouty homolog 1 [52]. While this study established the importance of miR-21 in MSC differentiation, miR-21 has also been shown to enhance MSC adipogenesis by targeting sprouty homolog 2 (SPRY2) [53]. Some miRNAs may also have synergistic effects in bone regeneration by differentially affecting multiple cell types. miR-378 has been found to simultaneously enhance MSC osteogenic differentiation and capillary tube formation in human umbilical vein endothelial cells (HUVECs) in vitro, making it a promising candidate for bone regeneration [54].

Barriers to siRNA and miRNA delivery

While multiple siRNA and miRNA have shown success in clinical trials treating human diseases, with the first siRNA drug being approved for clinical use in 2018, no RNAi molecules have yet moved beyond preclinical models in the context of musculoskeletal tissue regeneration. The primary cause of failure for RNAi therapies is lack of efficacy due to the challenges of delivery, and the immaturity of RNAi as a therapeutic may be limiting applications to otherwise untreatable genetic conditions. However, the continuous evolution of sophisticated delivery systems coupled with greater understanding of gene regulatory networks is promising for the development of regenerative RNAi approaches.

The ultimate bottleneck to realizing the tremendous therapeutic potential of RNAi is the need for multifunctional delivery systems to overcome the inherently multifaceted barriers to intracellular siRNA/miRNA delivery [63]. As a practical clinical approach, systemic administration is used to easily deliver drugs; however, siRNA/miRNA are susceptible to rapid renal clearance and degradation by nucleases present in serum leading to half-lives of less than 20 minutes [64, 65]. This hurdle is not limited to systemic delivery, as nucleases are present throughout all human tissues, organs, and bodily fluids [66-68]. If siRNA/miRNA remain intact within the serum environment, diffusive constraints through dense, tortuous extracellular matrices, often composed of amphiphilic glycoproteins, further hinder target tissue delivery [69, 70].

Upon reaching target cells/tissues, the mammalian phospholipid bilayer presents a physicochemical barrier to cellular entry. The nucleic acids' negative charge, hydrophilicity, and relatively large molecular weight inhibit passive diffusion through the anionic lipid bilayer of the cell membrane [71-73]. If taken up by endocytosis, endosomal-lysosomal trafficking can result in degradation of RNAi via nucleases in the lysosomal compartment [71] and a failure to reach the cytosol. These numerous barriers to RNAi delivery necessitate multifunctional delivery systems in order to realize the full potential of siRNA and miRNA therapeutics for bone regeneration and beyond.

Mediating successful siRNA/miRNA delivery

Nucleic acid modifications for siRNA delivery

Nuclease-mediated degradation and stimulation of the innate immune system via siRNA and miRNA critically limits siRNA/miRNA efficacy. Therefore, a multitude of structural modifications to the RNA phosphodiester backbone and/or ribose sugar backbone have been devised to increase nuclease stability and limit off-target effects and have been extensively reviewed [74-76]. A database (siRNAmod) was recently established encompassing 4,894 chemically modified siRNA sequences that utilize 128 different modifications, all of which are experimentally validated [77]. The 2'-OH of the ribose sugar is an attractive site for modification because it is not required for RNAi [78] and several nucleases catalyze RNA degradation via nucleophilic attack at this position [75]. The most common modifications made to the 2'-OH of RNA include 2'-O-methyl (2'-OMe) and locked nucleic acids [74, 79]. These modifications also reduce the innate immune response by avoiding toll-like receptor (TLR) activation, which can be caused by unmodified double stranded RNA [80-82].

Although siRNA/miRNA structural modifications increase stability and immune tolerance, they do not address challenges associated with cellular uptake and endosomal escape required for cytoplasmic delivery. Direct conjugation to drug delivery systems is an established approach to enable siRNA/miRNA delivery. Early approaches modified siRNA with cholesterol or other lipophilic moieties to improve pharmacokinetics, serum stability, and cell-membrane binding [83-85]. Cholesterol-conjugated miR-29 mimic, developed by miRagen Therapeutics, Inc., is currently in clinical trials to test safety, tolerability, and pharmacokinetics for the treatment of scleroderma and fibroplasia through inhibition of extracellular matrix molecule expression [86, 87]. Various polymers and peptides have also been explored as direct conjugates and are reviewed elsewhere [88]. The most developed siRNA-conjugate delivery system used in multiple clinical trials has been developed by Alnylam utilizing N-acetylgalactosamine (GalNAc) ligands [63, 88, 89]. This technology has achieved preliminary success in treating liver diseases due to high affinity of GalNAc to asialoglycoprotein receptors expressed by hepatocytes [90]; however, GalNAc-conjugated siRNA targeting transthyretin (Revusiran) was halted in phase 3 clinical trials due to excess deaths [91]. Although siRNA-conjugate systems are simplistic and well defined, poor biodistribution results in predominant accumulation in the liver via the first pass effect, limiting these approaches for musculoskeletal and other applications.

Nanoparticles for siRNA delivery

The unique physicochemical properties of nanoparticles (NP), including size, shape, surface chemistry, and potential for diverse functionalization, have led to applications in myriad therapeutic approaches from diagnostics to drug delivery [92-94]. Specifically, siRNA and miRNA delivery have benefitted immensely from nanoparticle-based delivery approaches [95, 96], highlighted by the first approval of an siRNA-based therapy in 2018, which is a NP formulation known as Patisiran [3]. Most commonly, NPs for siRNA delivery contain a cationic component that serves to complex and protect anionic nucleic acid molecules from nuclease degradation and allow for interaction with negatively charged cell membranes to

facilitate cellular uptake [97]. However, as endocytosis is the typical mechanism of nanocomplex cellular uptake, escape from endolysosomal trafficking is another delivery hurdle often addressed via NP delivery systems [98]. Thus, cytoplasmic delivery is typically achieved through moieties that enable endosomal escape through either the proton sponge effect or pH-dependent membrane destabilization.

Many materials have been utilized to form NP-siRNA/miRNA complexes and can be grouped into four main classes: lipid-based NP (LNP) systems [99], natural polymers [100], synthetic polymers [101], and inorganic nanoparticles [102, 103]. LNPs, which were previously developed for gene delivery, are the most commonly used non-viral vectors for delivery of siRNA and miRNA due to early realization of favorable pharmacokinetic properties such as long circulation time, serum stability, and biocompatibility [99, 104, 105]. These properties confer advantages specific to systemic administration and thus LNPs have been used to treat liver diseases [63] and a wide range of cancers [106, 107]. Although promising, LNPs have shown limited clinical efficacy and, in preclinical testing, have yet to move beyond applications in the liver [63, 105]. Furthermore, LNP-mediated delivery of siRNA is highly inefficient due to endocytic recycling, leading to exocytosis of approximately 70% of delivered LNP-siRNA [108], highlighting the importance of endosomal escape.

Polymers provide an attractive alternative platform for the development of NPs for siRNA and miRNA delivery. Both natural and synthetic polymers can be synthesized with a variety of cationic components that allow for complexation with nucleic acids and interaction with the cell membrane. Chitosan, a polysaccharide, is the most widely utilized natural polymer for siRNA delivery owing to its cationic charge, biocompatibility, and biodegradability [109]. However, chitosan has inherent limitations such as poor water solubility at physiological pH, leading to NP instability and low buffering capacity, resulting in poor endosomal escape and inefficient siRNA delivery. Substantial improvements have recently been made by employing synthetic polymer modifications to chitosan to improve biocompatibility, solubility, and transfection efficiency [100, 110].

Poly(ethylenimine) (PEI) is a commonly employed synthetic polymer for NP-mediated delivery of siRNA. PEI is cationic, which allows for formation of NPs via electrostatic interactions with siRNA, and bestows endosomal escape through a combination of the proton sponge effect and polymer swelling upon protonation in acidic vesicles [96, 111]. However, the cytotoxicity and immunogenicity of PEI has been well documented in a multitude of cell types *in vitro* and *in vivo* [112-115]. This has directed the field to explore a variety of PEI modifications, such as poly(ethylene glycol) (PEG) [112] and poly(caprolactone) (PCL) functionalization [116], and alternative polymers to overcome these significant limitations [117, 118]. More recently, poly(β-amino ester)s, traditionally used for gene delivery, were adapted to deliver siRNAs and incorporate reducible moieties to confer biodegradability to avoid polymer accumulation and mitigate toxicity [119].

Polymeric micellar NPs provide distinct advantages for siRNA and miRNA delivery compared to the aforementioned polymers and can be formed using self-assembling amphiphilic diblock polymers. Self-assembled NPs offer exceptional advantages of simple

syntheses using a wide array of functional monomers coupled with core-shell architecture that provides the multi-functionality demanded by RNAi delivery systems [101, 120]. In particular, pH responsive polymers exploit local drops in pH via protonable and/or membrane interacting monomers to facilitate siRNA early endosomal escape, improving delivery efficacy and reducing innate immunogenicity [98, 121-123]. Additionally, core-shell architecture allows incorporation of siRNA and/or additional drugs in the hydrophobic core with electrostatically bound siRNA and/or drugs complexed to the shell, which can further be modified with functional ligands [124, 125]. This exquisite functional versatility makes polymeric NPs promising candidates for siRNA delivery vehicles.

Inorganic NPs are also promising platforms for RNAi delivery [102, 103, 126-129]. Inorganic NPs offer high surface to volume ratios for drug loading either via conjugation or electrostatic interactions. Similar to polymeric systems, the surface chemistry of inorganic NPs are highly tunable to enable the simple introduction of targeting or functional groups to promote RNAi complexation and endosomal escape [102]. Unique to inorganic NPs is the ease with which they can be imaged noninvasively due to their optical properties, thus enabling label-free monitoring of biodistribution [128, 130]. Though not completely exhaustive, the most promising inorganic NPs used for siRNA include gold, calcium phosphate, and mesoporous silica [103]. Of these, mesoporous bioactive glass is the only material reported for bone-specific siRNA delivery where it has been utilized to treat preosteoblast and marrow stromal cells, suggesting promising cytocompatibility [131].

Nanoparticle-protein interactions and the implications for siRNA delivery to bone

Despite significant promise, translation of NP drug delivery systems for bone or other tissues suffer from additional delivery challenges. In particular, non-specific protein adsorption (fouling) is a significant hurdle. After systemic delivery, protein adsorption results in mononuclear phagocytic system (MPS)-mediated clearance of up to 99% of injected NP dose [132]. Additionally, MPS clearance can also occur locally via tissue-resident or recruited macrophages. MPS clearance alters tissue biodistribution to favor the liver [133-136], potentiates immunogenicity [136, 137], and necessitates dose escalation to achieve therapeutic benefits. If NPs reach cells of interest, in bone or otherwise, NP-protein interactions reduce siRNA release from NPs resulting in reduced efficacy.

The phenomenon of protein adsorption to biomaterials has been known since seminal studies first published in 1962 [138]. When introduced to biological fluids, biomaterials, including NPs, encounter an expansive array of proteins and macromolecules that interact with and adsorb to the NP surface, drastically altering NP physicochemical properties and biological identity. The term “corona” was first proposed to describe this layer of adsorbed protein in 2007 [139], and since then research in this area has grown rapidly. Formation of the corona is highly dependent upon NP properties, including hydrophilicity, size, charge, crystallinity, surface instability, and electronic states, as depicted in Figure 4 [140]. The NP-protein corona can be classified as biphasic, consisting of a hard corona composed of proteins irreversibly adsorbed to NP surface with high affinity and typically existing at high

concentrations in the biological medium, whereas the soft corona is more amorphous and consists of loosely-associated proteins with lower affinities and concentrations [141]. While little is known about the soft corona, as this layer is typically desorbed when isolating NP-protein complexes from the biological media, the hard corona has been characterized using multiple quantitative analytical approaches [142]. An exemplary study [143] used a combination of LC-mass spectrometry and proteomics analysis to show that over 300 individual proteins could be identified on poly(styrene) NPs after just a 30-second incubation in human plasma. Furthermore, the repertoire of proteins significantly changes over time [143] and varies with NP size, charge, and surface functionalization [144].

Early studies investigating the biological effects of NP-protein corona in vivo showed that complement C3 proteins adsorbed to poly(D,L-lactic acid) NPs [145] and liposomes [146], increasing clearance via the MPS. Such opsonization is one of the most critical biological barriers to NP-mediated drug delivery, and is mediated by proteins adsorbed to the NP surface [147]. Moreover, adsorbed protein identity alone is insufficient to predict NP-MPS interactions, as demonstrated in a study that found BSA underwent conformational changes upon adsorption to poly(methacrylic acid) nanoporous particles that subsequently stimulated secretion of proinflammatory cytokines in macrophages [148]. These studies establish the NP-protein corona as a critical determinant of NP cytotoxicity and immunostimulation [149].

In addition to toxicity, NP-protein adsorption can drastically alter NP colloidal stability, yet the majority of NP physicochemical properties are characterized in protein free buffers, naively ignoring these critical NP-protein interactions [150]. While the formation of a protein corona can stabilize silver [151] and gold [152] NPs, the presence of serum proteins potentiates aggregation of various polymeric NPs [153, 154], and is mediated by IgG and fibrinogen for poly(styrene) NPs [155]. These differences are mechanistically attributed to steric interactions of albumin on the NP surface [152]. It is well established that NP-protein interactions significantly affect cellular uptake and drug delivery [143, 156, 157]. These effects are often ignored in NP-siRNA delivery studies, and the implications can be detrimental. For example, cationic NP-siRNA complexes were found to severely aggregate in the presence of low levels of serum, dramatically impacting siRNA delivery efficacy [158]. The negative impact of aggregation on siRNA efficacy manifested in three ways: reduced cellular uptake, reduced pH-dependent membrane disruption, and reduced NP-siRNA decomplexation [158]. Similar effects have been observed in cationic liposomes [159]. These results highlight the importance of studying and mediating NP-protein interactions to develop successful systemic siRNA delivery approaches.

Conclusions and Future Opportunities for RNAi-mediated Bone Regeneration

siRNA offers the potential for a fundamental shift in bone regeneration approaches owing to its remarkable capacity to alter cell behavior through highly specific gene translational silencing. While *in vitro* and pre-clinical *in vivo* studies of multiple gene targets have been extremely promising, delivery remains the fundamental challenge to the use of siRNA. Bone

is a particularly challenging target for systemic drug delivery, with even small molecule drugs achieving bone accumulation of less than 1% of the injected dose [160]. Existing drug delivery systems have demonstrated some success in local delivery of siRNA and miRNA to bone for acute injuries such as fracture or critical sized bone defects. However, to treat osteoporosis or low bone mass or non-unions/delayed unions, which are not identified until months after injury, systemic delivery approaches are critical. Existing systemic drug delivery systems suffer from serum protein adsorption, leading to rapid MPS-mediated clearance and limited accumulation in bone. Though significantly improved bone accumulation has been shown using bone targeting approaches [4, 161], successful adaptation of siRNA therapeutics for bone regeneration require additional developments in materials design to ensure bone versus off-target accumulation of RNAi therapeutics.

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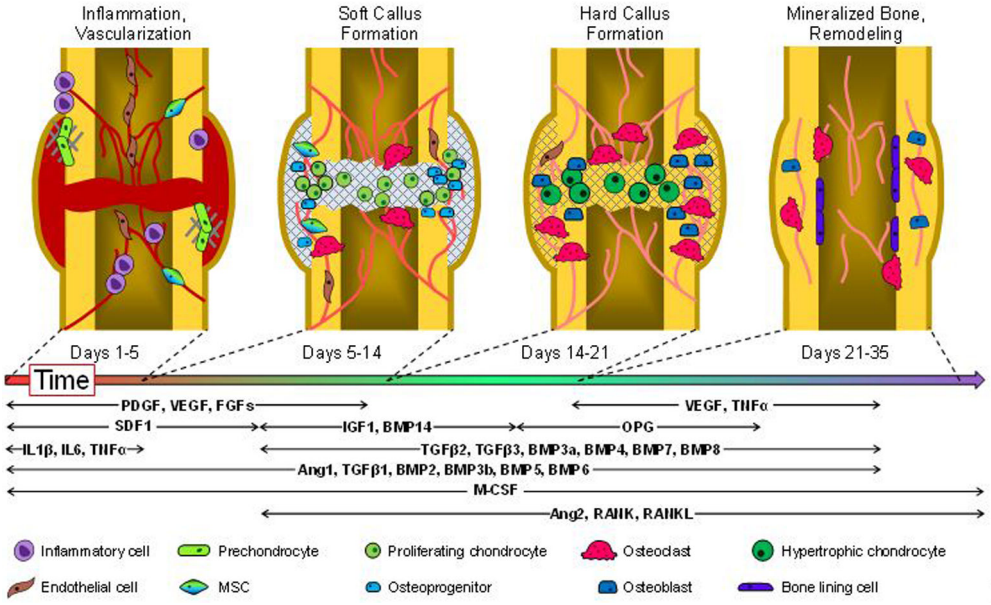

Figure 1.

A spatiotemporal cascade of multiple endogenous factors controls normal bone regeneration during fracture repair in four stages. PDGF = platelet derived growth factor; VEGF = vascular endothelial growth factor; FGF = fibroblast growth factor; TNF = tumor necrosis factor; SDF = stromal cell-derived factor; IGF = insulin-like growth factor; BMP = bone morphogenetic protein; OPG = osteoprotegerin; IL = interleukin; TGF = transforming growth factor; Ang = angiopoietin; M-CSF = macrophage colony stimulating factor; RANK = receptor activator of nuclear factor κB; RANKL = RANK-ligand. Reproduced from [4] with permission.

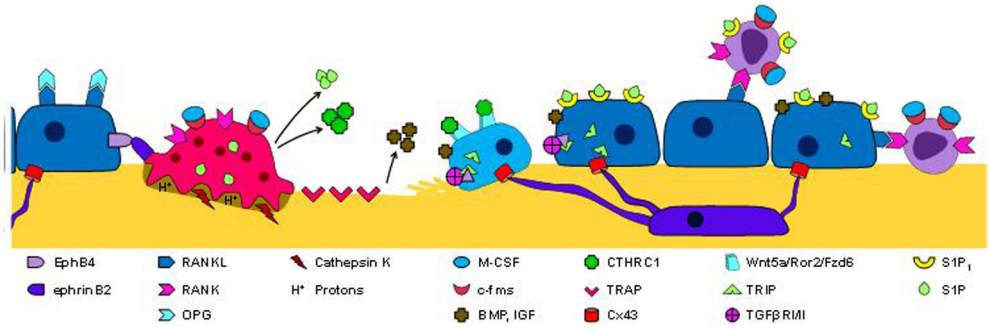

Figure 2.

Osteoblasts, osteoclasts, osteoprogenitors, osteocytes, and monocytes communicate through direct interactions and paracrine effects. RANKL = receptor activator of nuclear factor kappa B (RANK) ligand; OPG = osteoprotegerin; M-CSF = macrophage colony stimulating factor; BMP = bone morphogenetic protein; IGF = insulin-like growth factor; CTHRC1 = collagen triple helix repeat containing 1; TRAP = tartrate-resistant acid phosphatase; Cx43 = connexin 43; Wnt = wingless-related integration site; Ror = receptor tyrosine kinase-like orphan receptor; Fzd = frizzled; TRIP = transforming growth factor beta receptor (TGFβR)-interacting protein; S1P = sphingosine 1-phosphate; $S1P_1$ = S1P receptor.

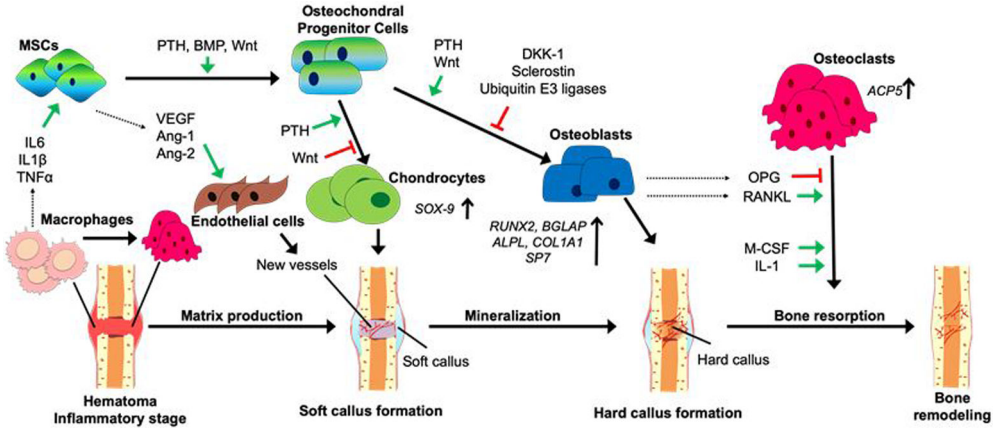

Figure 3.

Schematic of fracture healing, highlighting the various cells and factors that are negative and positive regulators of regeneration and highlighting potential targets for siRNA interventions. Abbreviations: ACP5 = acid phosphatase 5; ALPL = alkaline phosphatase; ANG = angiopoietin; BGLAP = bone gamma-carboxyglutamate protein (Osteocalcin); BMP = bone morphogenetic proteins; COL1A1 collagen type I, alpha 1; DKK1 = dickkopf-related protein 1; IL = interleukin; M-CSF = macrophage colony-stimulating factor; MSC = mesenchymal stem cell; OPG = osteoprotegerin; PPARG = peroxisome proliferator-activated receptor gamma; PTH = parathyroid hormone; RANKL = receptor activator of nuclear factor kappa-B ligand; RUNX2 = runt-related transcription factor 2; SOX-9 = sex determining region Y-Box 9; SP7 = osterix; TNFα = tumor necrosis factor alpha; VEGF = vascular endothelial growth factor; Wnt = wingless-related integration site. Adapted from [5] with permission.

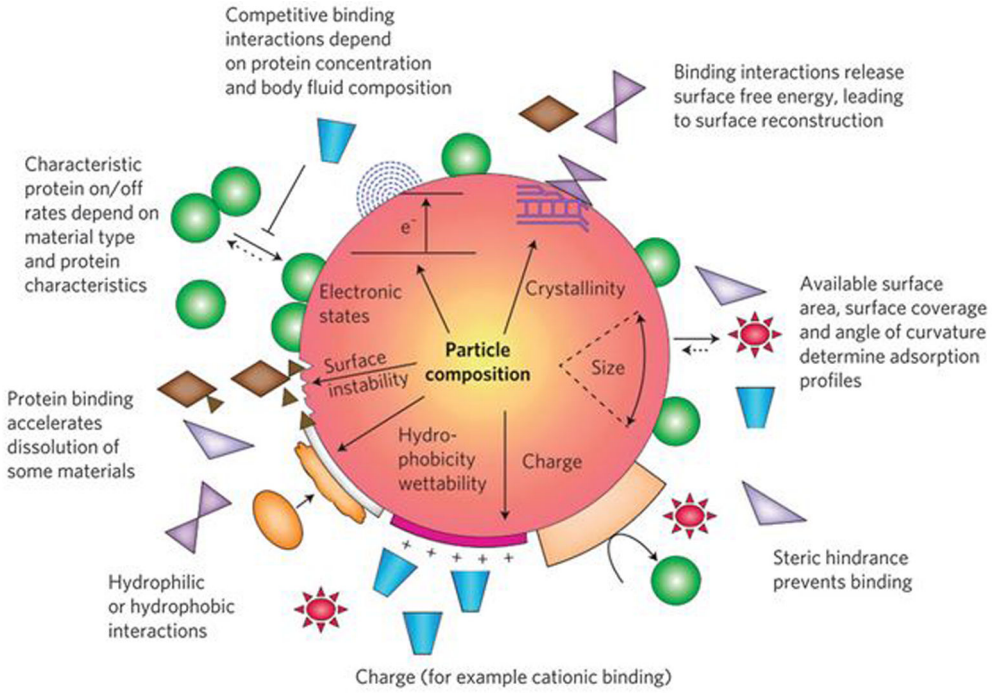

Figure 4.

Nanoparticle properties affecting the adsorption of proteins in vivo, which significantly effect biodistribution and delivery efficacy. Reproduced with permission from [140].

Table 1:

Examples of bone regenerative RNAi strategies

Target Pathway	Cell Target	Gene Targets	Molecule	Model Systems	Outcomes
Wnt	MSC	DKK1 [49]	miR-355-5p	In vitro	Activates Wnt-mediated osteogenic differentiation
RANK	Osteoclast	IKKγ [55], NFATc1 [56], Slfn2 [57], RANK [58]	siRNA	In vitro	Inhibited osteoclastogenesis
BMP	MSC	Noggin [59]	siRNA	Mouse calvarial defect	Enhanced bone regeneration
E3 Ubiquitin Ligase	MSC	Wwp1 [46]	siRNA	Mouse femur fractures	Enhanced bone regeneration
Other	MSC	GNAS1 [50]	siRNA	In vitro	Induces osteogenic differentiation
	MSC	Hdac5 [51]	miR-2861	In vitro	Promotes BMP2-related osteoblast differentiation
	Osteoblast	Sema4d [60, 61]	siRNA	OVX Mouse	Enhanced bone density
	MSC & EC	SuFu & Fus1 [54]	miR-378	In vitro	MSC osteogenesis and HUVEC capillary tube formation
		Runx2 & VEGF [62]	miR-26a	Mouse calvarial defects	Enhanced bone regeneration

Abbreviations: DKK1 = dickkopf-related protein 1; MSC = mesenchymal stem cell; RANK = receptor activator of nuclear factor kappa-B; RUNX2 = runt-related transcription factor 2; VEGF = vascular endothelial growth factor; Wnt = wingless-related integration site; IKKγ = inhibitor of nuclear factor kappa-B kinase subunit gamma; NFATc1 = Nuclear Factor Of Activated T Cells 1; Slfn2 = Schlafen-2; WWP1 = WW domain-containing E3 ubiquitin protein ligase 1; Hdac5 = histone deacetylase 5; OVX = ovariectomized; GNAS1 = guanidine nucleotide binding protein alpha stimulating activity polypeptide 1; Sema4d = Semaphorin4d; EC = endothelial cell; SuFu = Suppressor of fused homolog; Fus1 (or TUSC2) = Tumor suppressor candidate 2.