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## RNA therapeutics targeting osteoclast-mediated excessive bone resorption

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### Abstract

RNA interference (RNAi) is a sequence-specific post-transcriptional gene silencing technique developed with dramatically increasing utility for both scientific and therapeutic purposes. Short interfering RNA (siRNA) is currently exploited to regulate protein expression relevant to many therapeutic applications, and commonly used as a tool for elucidating disease-associated genes. Osteoporosis and their associated osteoporotic fragility fractures in both men and women are rapidly becoming a global healthcare crisis as average life expectancy increases worldwide. New therapeutics are needed for this increasing patient population. This review describes the diversity of molecular targets suitable for RNAi-based gene knock-down in osteoclasts to control osteoclast-mediated excessive bone resorption. We identify strategies for developing targeted siRNA delivery and efficient gene silencing, and describe opportunities and challenges of introducing siRNA as a therapeutic approach to hard and connective tissue disorders.

### Keywords

siRNA; osteoclast; molecular target; bone resorption; osteoporosis; gene therapy; RNAi

### 1. Introduction

Current treatments for osteoporosis can be divided into two categories: antiresorptive modulators and anabolic therapies. Estrogen, estrogen receptor modulators, calcitonin and bisphosphonates fall into the first category. Only one anabolic agent—teriparatide—is clinically approved, administered by daily injection.[1] The limited number of options in antiresorptive therapy reflects inadequate efficacy, severe side effects, high dosing frequency or low patient compliance:[1–6] estrogen treatment exhibits known side effects on breast and uterus inappropriate for long-term use; long-term calcitonin treatment can induce tolerance since more than half of the patients produce circulating antibodies to calcitonin. [7] Bisphosphonates, the most common current osteoporosis treatment, exhibit general side effects including gastrointestinal irritation, bone/joint pain and jaw osteonecrosis.[8–10] The latter two adverse effects can persist and even spread to new areas of unaffected bone after

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bisphosphonate therapy is discontinued. Because of bisphosphonate's long half-life, e.g., alendronate's half-life in bone is 10.9 years,[11] drug can remain active in bone for long periods after drug therapy is discontinued. Severe suppression of bone turnover has also been noted.[12, 13] Long-term bisphosphonate therapy increases fracture risk, including atypical fractures first reported in 2005.[14, 15] Recently, oral bisphosphonates were associated with 23 cases of esophageal cancer.[16] Therefore, maximum treatment duration for bisphosphonates is suggested to be five years.[17] Bisphosphonates are consequently counter-indicated in the context of fragility fracture healing. This enduring drug bioactivity presents substantial challenges for treatment of osteoporosis. Moreover, clinical studies of systemic bisphosphonate administration in combination with implants for 6 months or 2 years showed significant bone loss in the peri-implant area within three months post-operation.[18, 19] Similarly, in total hip arthroplasty, up to 14% bone loss was reported during the first three months post-surgery.[20]

Human clinical experience indicates that improved therapeutic strategies are needed in the context of osteoclastic bone resorption therapies. In 2010, denosumab (Prolia™, Amgen), a fully humanized monoclonal antibody targeting receptor activator of nuclear factor-kappa B ligand (RANKL), was approved by the FDA as a twice-yearly subcutaneous injection for treating postmenopausal osteoporosis. This antibody therapeutic appears well-tolerated and superior to the common bisphosphonate drug, alendronate, in preserving bone mineral density in several clinical trials to date.[21, 22] Additional new therapeutics are actively under investigation to meet the requirements of this fast-growing patient population worldwide.

## 2. RNA interference and siRNA delivery as a new therapeutic approach

RNA interference (RNAi) is a sequence-specific post-transcriptional gene silencing tool.[23] The process of gene-specific silencing through destruction of its mRNA transcript can be triggered by endogenous or exogenous small interfering RNAs (siRNAs).[24] Long double-stranded mRNAs derived from endogenous gene transcription or transfected transgene plasmids present in the cytoplasm can trigger the cleavage activity of the intracellular enzyme, Dicer, to cut mRNA into 19-nucleotide pairs with two nucleotide overhangs at both 3'-ends, called small interfering RNA (siRNA).[25] These double-stranded siRNA pieces then incorporate into RNA-induced silencing complexes (RISCs) which have a catalytic core comprising Argonaute (Ago) family proteins.[26, 27] Ago-2 then cleaves and removes the siRNA sense strand and thereafter RISC becomes activated with the remaining antisense siRNA strand. The antisense siRNA strand guides RISC activity in the cytoplasm to cleave its targeted complementary mRNA molecules, resulting in down-regulation of the targeted gene and corresponding protein expression. The mechanism of RNAi operation in mammalian cells is shown in Figure 1. First introduced in 2001, RNAi has been exploited for its highly specific mechanism of mRNA transcript targeting, and as a target screening and validation tool for cell signaling studies of many types.

RNAi is used for investigating and elucidating mammalian gene function as an alternative to knockout techniques. Specifically, siRNA also shows great potential for future targeted therapeutics for gene-associated diseases. Gene silencing using siRNA has several advantages intrinsic to RNAi, such as its high specificity, intrinsic biological response [28] and more efficient and specific silencing effects with lower dosing requirements, compared to antisense-based gene silencing.[29]

However, as polyanions, synthetic or *in situ* transcribed siRNAs do not readily or reliably enter mammalian cells. The main challenge in developing siRNA therapies, like other nucleic acid therapeutics, is to deliver them specifically into targeted tissues or cells. Viral

and non-viral vectors have been employed to address siRNA cell transfection inefficiency, and non-viral delivery is typically achieved by cationic lipoplexing reagents.[24, 30] Viral vector-based delivery is consistently associated with vector-based short hairpin RNA (shRNA) production systems, a DNA-based strategy to encode and obtain host-synthesized shRNAs in situ. These shRNAs can be further intracellularly processed into siRNA by Dicer. Both methods have their advantages and disadvantages. Non-viral delivery uses siRNA directly to generate potent silencing effects; therefore, it is simple and controllable. However, single-dose siRNA silencing effects are transient (up to five days in dividing cells),[24] and lipid-based siRNA delivery complexes can be removed from circulation by the liver rapidly, and lack tissue/cell specificity. The viral vector-based shRNA strategy has the potential of being able to provide stable, enduring gene silencing. Gene therapy can in principle continuously generate siRNA. The major bottleneck of the viral vector is its well-known safety issues.[24] Nevertheless, while non-viral delivery avoids the pitfalls of viral vector delivery, including high viral toxicity, possible carcinogenicity, proven immunogenicity, and significant cost limitations,[31] it is extremely inefficient in targeting, transfection and expression. Because of the substantial challenges with reliable systemic siRNA delivery and targeting, almost all current clinical foci for siRNA-based therapeutics are based on local or topical siRNA therapeutics. Successful siRNA delivery approaches currently include ocular, respiratory, central nervous system, dermal and vaginal delivery where local dosing accesses target cell populations directly.[32–36] One largely unexplored delivery route is via implantable combination devices facilitating local siRNA delivery directly from medical implants to adjacent tissue sites.[37]

### 3. RNAi applications in new osteoporosis therapies

As a nucleic acid therapeutic precedent, DNA-based gene therapy has developed rapidly for musculoskeletal applications in the last two decades. The therapeutic approach has been introduced to various disease categories: osteogenesis imperfecta,[38] lysosomal storage disorders,[39] rheumatoid arthritis,[40, 41] osteoarthritis,[42] and osteoporosis.[43–45] Specific to osteoporosis, gene transfer strategies deliver genetic material, either using intravenous injection of viral vectors carrying osteoprotegerin (OPG) cDNA[44, 45] or local injection of interleukin-1 receptor antagonist cDNA-transduced cells.[43] Due to desirable short-term transgene expression without the need to closely regulate transgene expression, DNA-based gene therapy has recently produced progress in musculoskeletal tissue healing. In a rat critical size defect model in femurs, BMP-2 cDNA-transduced cells seeded into collagenous scaffolds showed better healing compared with use of recombinant BMP-2 protein directly.[46] The feasibility of intralesional injection of viruses carrying cDNA encoding osteoinductive genes has been demonstrated in both rabbit and rat segmental defect models.[47, 48] Gene transfer strategies have been developed for many applications for musculoskeletal healing, such as spine fusion, articular cartilage and meniscus, intervertebral disc, ligament and tendon.[49] As DNA-based transgene therapies continue to demonstrate the potential for treating musculoskeletal diseases, providing a solid foundation for developing siRNA-based approaches in this field.

Applications of RNAi to musculoskeletal therapies can target a large and increasing number of signaling cascades in several tissue types, primarily bone and cartilage. In addition, RNAi can be utilized in several therapeutic categories: inflammation, degeneration, and regeneration. RNAi use in the context of treating rheumatoid arthritis has been actively investigated to date.[50] Osteoporosis is less studied but represents a particularly interesting application for RNAi therapeutics, targeting a diverse number of possible pathways achieved by local delivery to fragility sites via bone augmentation strategies. Instead of complete gene knock-out, both site-specific and temporally selective control over cellular signaling activity are perhaps more appealing for developing new osteoporosis therapies.

FDA-approved denosumab demonstrates precedent success in this regard. The transient efficacy of siRNA means it can be turned off and on, facilitating this transient programmed benefit. Notably, siRNAs have new targets distinct from other drug classes, with their own unique characteristics: they interrupt intrinsic cellular pathways with high targeting specificity.

#### 4. Osteoclastogenesis and osteoclastic bone resorption

Normal bone is constantly replaced by resorption of old bone by osteoclasts and deposition of new bone by osteoblasts. Continuous bone turnover results in the adult human skeleton being completely replaced every 10 years.[51] This balance of bone turnover is tightly regulated in healthy individuals as shown in Figure 2. Osteoclasts residing at or near the bone surface are multinucleated phagocytic cells formed by the fusion of monocyte macrophage precursor cells.[52] They are responsible for resorbing bone, working together with osteoblasts (bone producing cells), and playing a central role in normal bone remodeling. Two cytokines - nuclear factor kappa B (NF- $\kappa$ B) RANK ligand (RANKL, also called TRANCE/OPGL/ODF) and macrophage colony-stimulating factor (M-CSF) [53, 54] - are essential to this process [55–57] as shown in Figure 3. Previously published evidence shows that osteoclast formation can be stimulated *in vitro* using co-cultures of bone marrow cells and stromal cells/osteoblasts expressing those two cytokines.[58–60] M-CSF interacting with its *c-fms* receptor provides signals necessary for precursor cell survival and proliferation by receptor binding on early osteoclast precursor cells.[61] RANKL is the key cytokine for promoting osteoclastogenesis. Interactions between soluble RANKL and RANK receptor on the surface of osteoclast precursors are essential for expression of osteoclast-specific genes,[62] bone resorption and survival of mature osteoclasts.[63] Furthermore, osteoclastogenesis is negatively regulated by OPG (or osteoclastogenesis inhibitory factor, OCIF), also expressed by stromal cells and osteoblasts.[64] OPG is a decoy receptor that competes with RANK for binding RANKL.[65] Osteoclast production can be blocked by over-expression of OPG, resulting in osteopetrosis in mice.[64, 66] In this RANKL/RANK/OPG regulatory axis, positive regulator RANKL and negative regulator OPG are coordinated through interaction with RANK to regulate normal bone formation and degradation.

RANK is therefore a central factor in this bone metabolic regulatory pathway. It is central to much of the osteoclast functional phenotype and to bone metabolic balance. Therefore, RANK was chosen as the knock-down target to suppress osteoclast-mediated bone resorption.[67] Selective knockdown of RANK in mouse bone marrow cells can significantly block formation of Tartrate Resistant Acid Phosphatase (TRAP)-positive cells with RANKL and M-CSF *in vitro*. Cell-cell fusion can be inhibited in RANK siRNA-transfected osteoclasts, consistent with the fact that RANKL stimulation is critical for cell fusion to osteoclasts.[68] Successful transfections have been seen in mature osteoclasts, and osteoclast-mediated bone resorption was dramatically inhibited by RANK siRNA using the pit formation assay *in vitro*.[67]

Activation of RANK by RANKL not only induces osteoclast differentiation and survival, but also leads to activation of bone resorption by mature osteoclasts. Once activated, differentiated osteoclasts move to the target matrix and attach to the bone surface. After attachment, an isolated extracellular microenvironment, called the sealing zone, formed between the interface of the underlying bone with osteoclast membranes, is actively generated by osteoclasts.[69] Osteoclasts polarize themselves to form a ruffled membrane adjacent to the bone surface which is their resorptive organelle in the sealing zone. Osteoclasts then acidify and dissolve the underlying bone matrix by pumping hydrogen ions mediated by a vacuolar H<sup>+</sup>-adenosine triphosphatase (H<sup>+</sup>-ATPase) through the ruffled

membrane into the sealing zone. Osteoclast intracellular pH is maintained by  $\text{HCO}_3^-/\text{Cl}^-$  exchange across cell surfaces other than the ruffled membrane area.[70] These ion transporting events result in an acidic pH of ~4.5 only within the resorptive microenvironment.[71] The mineral phase of bone is first digested under this acidic milieu, followed by degradation of the collagen-rich demineralized organic component of bone by released hydrolytic enzymes.[72] Bone matrix degradation proteins are taken up by osteoclasts through the ruffled membrane from the resorption lacuna and released through the basolateral membrane into the extracellular space.[73] After local bone resorption is complete at this site, osteoclasts detach from the bone matrix and migrate to a new site to begin the next functional cycle.

Because of this central role in bone processing, osteoclasts are naturally a major target for most studies seeking to suppress bone resorption. In this review, siRNA targets for osteoporosis are sorted into three categories: osteoclastogenesis, osteoclastic bone resorption activity and osteoclast survival, including other potential associated targets identified to date. In addition, progress in developing efficient target silencing, delivery issues, and emerging opportunities and challenges to exploit siRNA for therapeutic purposes are discussed.

## 5. Targets for siRNA in osteoporosis and bone metabolism

### 5.1. Osteoclastogenesis

Transcription factors essential for osteoclastogenesis, including NF- $\kappa$ B downstream of RANK,[74] mediate osteoclast differentiation and inflammatory osteolysis.[75] Osteoclast differentiation failure is reported to be caused by mutant NF- $\kappa$ B in mice.[76] Its upstream regulator, I $\kappa$ B kinase (IKK) complex, contains IKK $\alpha$  and IKK $\beta$  as catalytic subunits and IKK $\gamma$  as a regulatory subunit.[77, 78] IKK $\alpha$  and IKK $\beta$  are required for normal bone homeostasis. IKK $\beta$  is essential for osteoclastogenesis and osteoclast survival since deletion of IKK $\beta$  impairs osteoclast differentiation in vitro and in vivo.[79, 80] IKK $\alpha(-/-)$  mice did not show overall skeletal defects, but IKK $\alpha(-/-)$  hematopoietic cells failed to differentiate into multinucleated osteoclasts.[81] Nonetheless, the role of IKK $\gamma$  is unclear. Recently, transient transfection of siRNA to inhibit IKK $\gamma$  in bone marrow-derived osteoclast precursors was reported using a retroviral delivery approach.[82] When IKK $\gamma$  was knocked down, formation of multinucleated osteoclasts was reduced 83% compared with controls. In addition, the role of IKK $\gamma$  was confirmed through observation that RANKL-induced osteolysis was impeded by preventing oligomerization of IKK $\gamma$  monomers using peptides in mice.[82] The study suggested that IKK $\gamma$  is essential for osteoclastogenesis, and thus, all three IKK subunits could be potential targets for inhibiting osteoclastogenesis and osteolysis using siRNA.

Interactions between RANKL and RANK are essential for osteoclastogenesis. RANK has three TNF receptor-associated factor 6 (TRAF6) binding sites and recruits TRAF6 to activate the transcription factors NF- $\kappa$ B and activator protein-1 (AP-1).[83, 84] Nuclear factor of activated T cells (NFATc1) has been demonstrated as the transcription factor most strongly induced by NF- $\kappa$ B activation.[85] NFATc1 is also known to be important in immune response and also play an essential role in osteoclastogenesis.[85, 86] Therefore, siRNA targeting NFATc1 in both RAW 264.7 cells and RAW-induced osteoclasts under LPS stimulation was examined.[87] Specific knockdown of NFATc1 led to reduced formation of mature osteoclasts and significantly decreased osteoclast-specific gene expression, including TRAP and cathepsin K, both responsible for effective osteoclastic bone resorption.

Once RANK is activated by its ligand, several signaling cascades are induced during osteoclast formation or activation, resulting in activation of several transcription factors mediated by protein kinases,[88] such as c-Fos, NF- $\kappa$ B, c-Jun, NFATc1.[84] Transcription factor c-Jun has recently shown its essential role in osteoclastogenesis.[89] Interaction between RANK and RANKL can stimulate JNK and activate c-Jun which complexes with c-Fos downstream to form AP-1.[84] Moreover, c-Jun was reported to regulate osteoclastogenic activities of NFATc-1: dominant-negative c-Jun transgenic mice were unable to form osteoclasts due to arrested activation and expression of the NFAT family proteins.[90] Recently, using a proteomic approach, a member of a family of growth regulatory genes, schlafen2 (Slfn2), was found to be highly induced by RANKL activation and capable of regulating c-Jun activation.[91] Using RNA silencing technology, knockdown of Slfn2 resulted in significantly reduced expression of c-Jun and NFATc1 and 50% less TRAP-positive multinucleated cells compared with control. Furthermore, over-expression of Slfn2 enhanced c-Jun phosphorylation. Slfn2 therefore functions downstream of RANK/RANKL signaling and upstream of c-Jun and NFATc1 in osteoclastogenic regulation.

Dendritic cell-specific transmembrane protein (DC-STAMP), a seven subunit-transmembrane protein originally found in dendritic cells [92] is highly expressed in osteoclasts but not in macrophages.[93] Expression levels of DC-STAMP can be rapidly up-regulated under RANKL-mediated osteoclastogenesis in osteoclast precursors.[94] Using siRNA to specifically knock down its expression in RAW-D cells completely abrogated the formation of multinucleated osteoclast-like cells stimulated with RANKL and TNF- $\alpha$  in vitro.[94] Consistent with this, osteoclastogenesis can also be enhanced by over-expression of DC-STAMP and vice versa.[94] In addition, DC-STAMP has been further proven essential for both osteoclast and foreign body giant cell fusion. [93] No multinuclear osteoclasts were observed in bone sections from DC-STAMP-deficient mice, and their mononuclear cells were unable to differentiate to multinuclear osteoclasts under stimulation with RANKL and M-CSF in vitro, remaining mononuclear, TRAP-positive cells. [93] These cells are able to resorb bone, but with much lower efficiency, exhibiting the pathogenesis of moderate osteopetrosis.[93] Consistent with these results, bone mineral density and bone volume per tissue volume in DC-STAMP<sup>-/-</sup> mice increased compared with wild type controls.[93] DC-STAMP is therefore concluded to be indispensable for osteoclast multinucleation.

A novel gene bearing significant similarity to the DC-STAMP family, called osteoclast stimulatory transmembrane protein (OC-STAMP), was identified in both primary bone marrow cells and RAW264.7 cells in 2007.[95] It is strongly up-regulated in response to RANKL stimulation as a multiple transmembrane protein in osteoclasts. Blocking OC-STAMP expression by either RNA interference or OC-STAMP antibodies showed similar results as blocked DC-STAMP: mononuclear, TRAP-positive cells.[95] The results suggest that both DC-STAMP and OC-STAMP are responsible for cell fusion and each of them can not compensate for the other since knock-down of only one of them impaired osteoclast multinucleation.

A study aimed at protecting biomaterials by inhibiting macrophage fusion at implant sites showed that using siRNA targeting Rac1 can limit fusion without limiting phagocytosis.[96] As the study attempted to find the fusion target shared by foreign body giant cells formed by monocyte fusion and also by osteoclasts, osteoclast formation theoretically should be inhibited as well. Further study is required to confirm the effect of Rac1 silencing on monocyte fusion to osteoclasts.

A membrane glycoprotein, CD9, one of the tetraspanins, has been reported to play an important role in cell fusion during osteoclastogenesis.[97] CD9 has been reported to be involved in cell-cell fusion and cell motility in different cells.[98, 99] In RAW 264.7 cells, CD9 localizes to the cell membrane and its expression is up-regulated by RANKL stimulation. [97] Targeted inhibition of CD9 using siRNA is able to significantly inhibit the formation of RAW 264.7 differentiated osteoclasts by inhibiting cell fusion in cultures. Over-expression of CD9, interestingly, can promote cell fusion in RAW 264.7 cultures without RANKL stimulation, but fused cells are TRAP-negative. In the same study, CD9 is confirmed to be expressed in osteoclasts *in vivo* by immunohistochemical analysis of tissue sections from mouse femoral bone.

During the osteoclastogenesis cell-cell fusion process, sialic acid which is involved in a number of biologic responses, was hypothesized to play a role in this process.[100] Takahata et.al.[101] found that alpha(2,6)-linked-sialic acid degraded during osteoclast differentiation and desialylated cells only could form mononuclear TRAP+ cells with normal expression of osteoclast markers. Using siRNA to knock down alpha(2,6)-sialyltransferase produced significant inhibition of osteoclast multinucleation, which suggests a role for alpha (2,6)-linked-sialic acid in cell-cell fusion processes during osteoclastogenesis.

A novel gene, *nha-oc/NHA2* is significantly upregulated in RANKL-stimulated osteoclast precursors.[102] As the murine orthologue of the human gene *HsNHA2*,[103] encoding a cation-proton antiporter (CPA) localized on the mitochondria, it is proposed to regulate proton concentration in osteoclast mitochondria. *NHA-oc/NHA2* selectively expressed in differentiated osteoclasts regulates Na<sup>+</sup>-dependent mitochondrial pH changes and mitochondrial passive swelling. Importantly, *nha-oc/NHA2* siRNA inhibits TRAP-positive multinucleated cell formation and resorption activity under RANKL stimulation. This reduction partially resulted from apoptosis induced from the loss of inhibition of caspase-9 activation by *NHA-oc/NHA2*. Therefore, *NHA-oc/NHA2* is integrally involved in osteoclast formation, resorption and survival.

Ovarian cancer G-protein-coupled receptor 1 (OGR1) is a histidine-enriched proton-sensor. The OGR1 gene was found to be 7-fold up-regulated in the long bone of CSF-1-null osteopetrotic rats after CSF-1 injections compared to untreated mutants.[104] Expression of OGR1 can also be induced in RAW 264.7 cells under RANKL stimulation during osteoclast differentiation. Specifically knocking down OGR1 expression by siRNA (>1µg/ml) produced almost 50% inhibition of osteoclast formation in both mouse bone marrow mononuclear and RAW 264.7 cells without significant cell death. Concomitant with confirming OGR1's role in regulating osteoclastogenesis, the expression and function of the regulators of G-protein signaling (RGS) proteins of osteoclastogenic RANKL signaling cascades were investigated. RGS proteins have been suggested to physiologically regulate G-protein cycles and G protein signaling in hematopoietic cells.[105, 106] In this protein family, RGS18, specifically expressed in hematopoietic cells, showed consistent decreases in mRNA expression levels during RANKL stimulated osteoclastogenesis.[107, 108] Target-specific knockdown of RGS18 in RAW264.7 cells using siRNA resulted in enhanced osteoclast formation under RANKL induction. Additionally, antibodies against OGR1 with Zn<sup>2+</sup> ion addition (antagonist of OGR1)[109] significantly reversed the effects of RGS18 siRNA. These observations suggest that the ability of RGS18 to suppress osteoclastogenesis depends on the OGR1 signaling pathway and inhibition of OGR1-mediated cell signaling. [108]

Another RGS member, RGS12, is reported to be significantly upregulated after RANKL stimulation, with its expression being dose-dependent on RANKL.[110] Using vector-based RNAi technology, stable RGS12-silenced RAW 264.7 cells showed 18.7 times lower

numbers of TRAP-positive multinucleated cells under RANKL stimulation compared with control groups. The mechanism of completely blocking osteoclast differentiation was suggested to lie in regulation of intracellular  $\text{Ca}^{2+}$  oscillations in the NFAT2 pathway during differentiation. Such  $\text{Ca}^{2+}$  oscillations can be completely blocked and the expression of NFAT2 simultaneously significantly inhibited in RGS 12-silenced cells.[110]

Triggering receptor expressed in myeloid cells-1 (TREM2) is produced in myeloid cells in bone marrow, and locates to these cell surfaces. Interactions between TREM2 and DAP12 produced from the TYROBP gene transmit signals to activate the cells. Osteoclast precursor cells harvested from DAP12<sup>-/-</sup> mice only form mononuclear TRAP-positive cells under stimulation with RANKL and M-CSF. These cells exhibit only 50% ability to resorb bone compared with wild type cells.[111] siRNA against TREM2-transfected RAW264.7 cells largely reduces numbers of TRAP-positive cells, and most were small (<10 nuclei) or mononucleated cells. Thus, TREM2/DAP12 signaling plays an important role in osteoclast differentiation under RANKL stimulation.[112] The same study blocked TREM2 in murine primary cell-induced osteoclasts using antibodies and showed decreased bone resorption, providing evidence that the TREM2/DAP12 signal also regulates osteoclast function.

Extended space flight can cause severe bone loss in astronauts due to microgravity. Compared with normal gravity, microgravity can accelerate osteoclastogenesis about 2-fold.[113] Gene expression profiling of RAW 264.7 cells under microgravity conditions has shown increased expression of the calcium-binding protein A8/calgranulin A (S100A8). siRNA knockdown of S100A8 in RAW 264.7 cells significantly suppressed osteoclastogenesis under microgravity conditions. S100A8 could be a therapeutic target for preventing bone loss during extended space flights.

Retinoblastoma protein-interacting zinc finger 1 (RIZ1) protein also participates in RANKL-induced osteoclastogenesis.[114] binding with both retinoblastoma protein and estrogen receptors and reportedly involved in osteosarcoma.[115–117] RIZ1 expression increases under RANKL induction at 24 hours, and RIZ1 siRNA-transfected RAW 264.7 cells showed significantly inhibited NFATc1 activation 3 days post-RANKL and M-CSF treatment, but with no significant influence on TRAF6 expression. Thus, RIZ1 is suggested to positively regulate NFAT-1 activity at the last stages of osteoclastogenesis. RAW 264.7 cells with reduced RIZ1 expression exhibited substantially reduced ability to form TRAP-positive multi-nucleated osteoclast-like cells under RANKL and M-CSF stimulation. Recently, c-Abl SH3 domain-binding protein-2 (3BP2) has been recognized as a key regulator of RANKL-induced osteoclastogenesis.[118] 3BP2 regulates several immunoreceptor signaling pathways in immune cells, such as T, B, and NK cells, and was reported to promote activation of NFAT in T and B cells.[119–123] Knockdown of 3BP2 in RAW 264.7 cells decreased expression of NFATc1 and completely suppressed RANKL-induced TRAP-positive multinucleate cell formation.[118]

The family of disintegrin and metalloproteinases (ADAM) peptides are cell surface proteins involved in cell adhesion and cell fusion.[124] ADAM8 is a transmembrane glycoprotein expressed in monocytes and significantly up-regulated during osteoclastogenesis.[125, 126] Recently, ADAM8 has been shown to promote osteoclastogenesis whereby vitamin 1,25-(OH)<sub>2</sub>D<sub>3</sub>-stimulated osteoclast formation was inhibited by ADAM8 antisense treatment.[126] Its direct effect on osteoclastogenesis was also investigated using siRNA.[127] Silencing ADAM8 in RAW264.7 cells decreased osteoclast formation and cell size compared with controls, and modestly decreased osteoclast marker mRNA levels when stimulated with RANKL. In contrast, transfection of ADAM8 into RAW264.7 cultures increased osteoclast marker mRNA expression levels and increased the number of TRAP-positive cells. The study also showed that ADAM8 was highly expressed in rheumatoid arthritis (RA) pannus



macrophages and multinucleated cells adjacent to eroded cartilage. Therefore, ADAM8 was a recommended target for suppressing RA progression by preventing osteoclast formation.

Epidermal growth factor receptor (EGFR) has been the focus of increasing attention in osteoclastic bone resorption, shown to promote bone resorption *in vitro*. [128] Its inhibition *in vitro* resulted in suppression of bone marrow stromal cell induced osteoclast differentiation. [129] Yi et al [130] investigated EGFR function in osteoclast formation and survival, and found that EGFR expression was highly up-regulated by RANKL. Knockdown of EGFR in bone marrow monocytes using lentivirus expressing shRNA completely suppressed osteoclastogenesis with RANKL and M-CSF. In addition, EGFR siRNA blocked NFATc1 expression completely under RANKL stimulation. This study also showed that EGFR co-immunoprecipitated with RANK and Gab2 which mediates the RANK signaling cascade. These data imply that EGFR may couple with RANK via the signaling mediator Gab2 to regulate RANK-stimulated downstream pathways, playing an important role in osteoclastogenesis.

Multinucleated osteoclasts are formed by fusion of monocyte macrophages. Therefore, the role of integrins important for trafficking monocytes in osteoclastogenesis has been assessed using siRNA. Only two integrin pairs are found on pre-osteoclast monocytes: CD11a/CD18 (LFA-1), and CD11b/CD18 (Mac-1) which is considered the premier marker of pre-osteoclasts. [131, 132] Antibodies against CD11b and CD18 to block Mac-1 both in RAW264.7 and primary cell culture can significantly inhibit osteoclastogenesis, but not antibodies against CD11a. [133] Specific knockdown of CD11b using siRNA in RAW 264.7 cells, yielding a 50% decrease in CD11b expression, resulted in ~50% reduction in osteoclast area. This inhibition was suggested to occur in the early stages of cell differentiation since it was accompanied by a three-fold increase in the mRNA expression level of NFATc1, a major regulator of osteoclastogenesis. [133] Taken together, integrin Mac-1 is suggested to play an important role in early stage osteoclast differentiation via pre-osteoclast cell-cell interactions.

A new member of the TNF family that induces cell apoptosis, called TNF-related apoptosis-inducing ligand (TRAIL), shares a 25% amino acid homology with RANKL [134] and is reported to inhibit osteoclastogenesis in both human peripheral blood mononuclear cells (PBMC) and RAW264.7 cells, possibly by inhibiting the p38/MAPK pathway. [135] It is also involved in human osteoclast apoptosis. [136] More recently, TRAIL has been found to inhibit the accumulation of RANKL-dependent p27<sup>Kip1</sup> in PBMC. [137] p27<sup>Kip1</sup> is a cyclin-dependent kinase inhibitor shown to be progressively upregulated to play an important role in mediating RANKL-induced murine osteoclastogenesis. [138, 139] Addition of TRAIL into pre-osteoclast cultures incubated with RANKL and M-CSF resulted in the 6-fold decrease in formation of TRAP-positive cells and significant reduction in the expression of p27<sup>Kip1</sup>. [137] Using siRNA to specifically knock down p27<sup>Kip1</sup> in PBMC culture dramatically reduces formation of TRAP-positive cells induced by RANKL and M-CSF, consistent with the results of p27<sup>Kip1</sup>-deficient mice. [139] Taken together, p27<sup>Kip1</sup> function alone appears essential for RANKL-mediated osteoclastogenesis.

## 5.2. Bone resorption targets

After osteoclasts mobilize the bone mineral phase, several hydrolytic enzymes degrade the organic bone component. The principal protease involved in this process is cathepsin K, [72, 140] a lysosomal protease mainly expressed in osteoclasts and released from the ruffled membrane to the resorption lacunae in the sealed zone. Cathepsin K mRNA and protein expression levels are stimulated by RANKL in a time- and dose-dependent manner, and increase with osteoclast differentiation and activation. [141–143] Cathepsin K efficiently degrades type I and II collagen, and gene mutation can cause osteopetrosis and exhibit

features of pycnodystosis.[140, 144, 145] Specific down-regulation of cathepsin K mRNA expression decreased both the number and area of bone pits more than 50% without significantly changing cell viability.[146] The unchanged osteoclast number may benefit maintenance of bone homeostasis since osteoclast and osteoblast activities are closely correlated, and disruption of this communication may cause other issues.[147] In mature osteoclasts, cathepsin K expression is increased by RANKL, both in vitro and in vivo.[141] RANK-RANKL binding activates at least five distinct downstream signaling pathways. [148] TRAF6 acts as a critical adaptor in binding with the cytoplasmic domain of RANK; its mutations cause osteopetrosis.[149, 150] Activation of transcription factor AP-1 has been suggested to result from TRAF6 signaling.[143] AP-1 is a heterodimeric protein composed of Jun (*c-Jun*, JunB or JunD) and Fos.[151] Transfecting RAW 264.7 cells with either the dominant negative form of *c-fos* or siRNA against either *c-jun* or JunB down-regulated RANKL-mediated cathepsin K gene expression. Both transfections inhibited CTSK promoter activity, suggesting that AP-1 stimulated the cathepsin K promoter.[143] In addition, c-Fos is known to play a critical role in osteoclastogenesis: over-expression of c-Fos rescues RANKL-induced osteoclast formation previously blocked by *N,N*-dimethyl-*D*-erythro-sphingosine treatment.[152]

H<sup>+</sup>-ATPase in the osteoclast ruffled membrane is responsible for reducing and maintaining the low pH in the sealed resorption lacuna during the process of osteoclast-mediated extracellular acidification. The ATPases have multiple subunits. One subunit isoform located in the ruffled membrane, subunit a3, is reported to be highly expressed and essential to osteoclast resorption function. Defects in this subunit of the vacuolar proton pump induce severe osteopetrosis.[153, 154] Hu et al.[155] showed successful carrier-free a3 siRNA transfection of primary rat osteoclast cultures, reporting that actin ring structures characteristic of actively resorbing osteoclasts were reduced to 20% of controls after siRNA transfection, but re-emerge after halting transfections. Bone resorption pits were significantly reduced 48 hrs after transfections and type I collagen C-terminal cross-linked telopeptides in the culture media were decreased more than 50% compared to controls or non-targeting siRNA treated groups. H<sup>+</sup>-ATPase knockdown is similar with cathepsin K knockdown: specific gene down-regulation reduces osteoclastic resorption activity without inducing cell death.

H<sup>+</sup>-ATPases have two domains: V1 located on the cytoplasm side and V0 bound within the membrane.[156] ATP-hydrolytic domain V1 has 8 individual subunits, A-H.[157, 158] Subunit C in murine H<sup>+</sup>-ATPase has three isoforms: Atp6v1c1 (C1), Atp6v1c2a (C2a), and Atp6v1c2b (C2b).[159] Only C1 is highly expressed in osteoclasts compared to the other two, and is highly up-regulated after RANKL and M-CSF stimulation during differentiation. [160] Feng et al.[160] depleted C1 expression in murine bone marrow macrophages with no significant difference in numbers of TRAP-positive multinucleated cells compared with untreated cultures. However, osteoclastic ability to actively produce H<sup>+</sup> to acidify the extracellular sealing zone environment was largely impaired, and areas of resorption pits on dentin slices by siRNA-treated osteoclasts were 0.5~0.75% of control cultures. Co-immunoprecipitation studies showed that C1 interacted with another essential ruffled border H<sup>+</sup>-ATPase subunit a3 and co-localizes with microtubules, but distinct from a3 deficiency, [62] C1 depletion was able to impair F-actin ring formation.[160] C1 was therefore suggested to be a regulator of F-actin sealing ring formation during osteoclast activation. Taken together, C1 knockdown does not affect osteoclastogenesis, but inhibits osteoclastic bone resorption.

Electrostatic neutralization of the membrane potential gradient during osteoclast acid secretion is maintained by Cl<sup>-</sup> ion channels on the osteoclast's ruffled border.[161] CIC-7 Cl<sup>-</sup> channels are highly expressed in osteoclasts and localize to the ruffled border

membrane. Disruption of CIC-7 in both humans and mice produced severe osteopetrosis. [161] With the exception of CIC-7, CIC-3 in the CLC family of channels was identified in osteoclasts as the functional Cl<sup>-</sup> ion-specific channel in intracellular organelles, such as endosomes and lysosomes. [162] Both organelle acidity and bone resorption activity are reduced using siRNA against CIC-3. Okamoto et al. concluded that observed osteoclastic bone resorption relied on such internal organelle acidification, since CIC-3 only locates to intracellular organelles, influencing this organelle acidity exclusively. However, bone resorption activity remains in the absence of CIC-3 activity in osteoclasts, attributed to the role of CIC-7 or other redundant Cl<sup>-</sup> ion channels. [162, 163] Since CIC-7 causes severe osteopetrosis in chloride channel-deficient mice, [161] the functional role of K<sup>+</sup>/Cl<sup>-</sup> co-transporters (KCCs) in mouse osteoclast-mediated bone resorption has been a focus. KCCs exist in many tissues and cells, transporting K<sup>+</sup> and Cl<sup>-</sup> ions each driven by their respective individual chemical gradients. [164, 165] A previous study showed that both CIC-7 and KCC1 mRNA was expressed in murine osteoclasts, with KCC1 locating to the cell membrane. [163] The KCC inhibitor, R(+)-butylindazole (DIOA) was able to increase the intracellular concentration of both Cl<sup>-</sup> and H<sup>+</sup> in resorbing osteoclasts. Thus, inhibiting KCCs can suppress Cl<sup>-</sup> secretion in resorbing osteoclasts, which eventually produces reduced H<sup>+</sup> pumping activity. Transfecting bone marrow cell-induced osteoclasts with siRNA against KCC1 dramatically reduces of the area of bone resorption pits compared with controls and these results are similar, but slightly less potent, to those from CIC-7 inhibition. [163]

The Na<sup>+</sup>/Ca<sup>2+</sup> ion exchanger (NCX) is a bi-directional membrane transporter regulating Ca<sup>2+</sup> homeostasis in many tissues on cell plasma membrane. [166] The NCX family comprises homologous proteins: NCX 1, NCX 2, NCX 3. [167, 168] In murine osteoclasts, three splice variants of NCX1 and NCX3, called NCX1.3, NCX1.41 and NCX3.2, were detected. [169] NCX mediates both Ca<sup>2+</sup> efflux and influx and is predicted to locate to the ruffled border and control Ca<sup>2+</sup> influx in resorbing osteoclasts. [169] SiRNA delivered against each NCX1.3, NCX1.41 and NCX3.2 at 100nM in mouse bone marrow cell-induced osteoclasts all significantly reduced the area of bone resorption pits per osteoclast approximately 50%. [169] These data support the essential role of these three NCX in Ca<sup>2+</sup> transport and their important role in regulating bone resorption.

The c-Src gene, encoding a cytosolic protein tyrosine kinase (PTK), [170] is also essential for osteoclast activity. Lack of c-Src expression in mice resulted in osteopetrosis characterized by inactive osteoclasts. [171] Previous work indicates that c-Src is functional as an adaptor in osteoclasts, regulating cell attachment and migration by recruiting essential signaling proteins. [172] Strong c-Src PTK activity has been found in ruffled borders in active osteoclasts. [173] Osteoclast-mediated bone resorption can be abolished both in vivo and in vitro by blocking c-Src PTK activity in osteoclasts. [174] Knockdown of Src expression in RAW 264.7 cells reduces both size and number of actin rings, and decreased cell spreading and fusion rates. [175] c-Src PTK activity is activated by dephosphorylation, and a structurally unique osteoclast-specific transmembrane protein-tyrosine phosphatase (PTP-oc) in osteoclasts is known. [176] RAW/C4 osteoclast-like precursor cells transfected with PTP-oc siRNA in serum-containing media and showed RANKL-mediated suppression of osteoclast differentiation. Both osteoclast number and size was significantly reduced by decreasing endogenous PTP-oc mRNA levels. [177] In addition, apoptosis was induced significantly by PTP-oc siRNA compared to control siRNA. Enhanced apoptosis from such knock-down also suggests its role in osteoclasts. Additionally, PTP-oc is also indicated to be involved in regulating RANKL-mediated osteoclastic differentiation. [178] A homologous recombination strategy was used to inhibit PTP-oc function in RAW264.7 cell culture. PTP-oc knockout cells could not form TRAP-positive multinucleated osteoclast-like cells under RANKL treatment after 7 days. Therefore, PTP-oc is a target not only on mature osteoclasts

to inhibit bone resorption, but also on osteoclast precursors to limit differentiation into osteoclasts.

The osteoclast sealing zone requires tight attachment between osteoclasts and the bone surface for successful bone resorption[179] and this depends upon formation of a dense belt-like actin ring surrounding the ruffled membrane. [180] These osteoclast actin rings have high concentrations of actin filaments assembled locally into dynamic structures as podosomes in bone attachment sites. With its significant prerequisite role in osteoclastic bone resorption, actin ring formation is a target for inhibiting osteoclasts and has been investigated in many studies. Gelsolin is an important actin regulator, necessary for podosome formation but not necessary for actin ring formation, which means that gelsolin-null osteoclasts are able to resorb bone, but with reduced potency.[181–183] However, Wiscott-Aldrich syndrome protein (WASP) critical for podosome assembly exists in the actin ring of gelsolin-null osteoclasts.[182] WASP interacts with phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>) and Cdc42 in response to integrin  $\alpha$ v $\beta$ 3 signaling, enhancing the sealing zone formation and bone resorption.[182] Mice without WASP expression exhibited defects in formation of podosomes and actin rings in the sealing zone and bone formation. [184] Attenuation of WASP using siRNA demonstrated the absence of podosome formation of actin rings and reduced bone resorption. WASP is activated by the coordinated binding of Cdc42 and PIP<sub>2</sub>, and full activation stimulates the actin-nucleating function of the actin-related protein (Arp)2/3 complex by associating with Arp2/3.[185–188] The Arp2/3 complex initiates actin nucleation and crosslinking, and has been reported to be 3-fold upregulated in RAW 264.7 cells in response to RANKL.[189, 190] Knock-down of Arp2 in RANKL-stimulated RAW 264.7 cells using siRNA generated an average 70% protein reduction 30 hours post-transfection.[190] Fewer podosome-like structures appeared, and compared with control, less than 1% actin rings were observed after knockdown, proving the vital role for Arp2/3 in actin ring formation and its potential to be a new target for therapeutic agents. Actin nucleation requires phosphorylation of WASP's C-terminal VCA domain during WASP binding to Arp2/3 complex after integrating a number of signals. [191–193] The PTP-PEST (protein-tyrosine phosphatase-proline, glutamic acid, serine, threonine amino acid sequence) has been shown to be involved in WASP and Src phosphorylation and dephosphorylation.[194] This regulates phosphorylation of proteins associated with WASP and enhances interactions between WASP, actin monomers, and the Arp2/3 complex by increasing the WASP conformational stability.[194] At the same time, it regulates Src activity through (de)phosphorylation.[195] Reducing PTP-PEST expression levels using siRNA in osteoclasts eliminated actin rings and significantly inhibited formation of the sealing zone, formation of WASP-cortactin-Arp2 complexes[195] and bone resorption.[194] This indicates a direct role of PTP-PEST in the formation of cell-sealing zones while interacting with WASP.

Simultaneously with WASP, cortactin, a c-Src substrate, binds with Arp2/3 at its N-terminal acidic domain to promote Arp2/3-induced actin assembly and stabilize actin filaments by binding to their repeat regions.[196] Cortactin was not found in hematopoietic cell precursors, but is induced in differentiated osteoclasts. Depletion of cortactin in primary mouse osteoclasts using siRNA resulted in the absence of podosomes, sealing rings and loss of bone-resorbing ability, suggesting an indispensable role for cortactin in osteoclastic bone resorption. Cortactin's mechanism for regulating sealing ring formation has been further studied.[197] Instead of inhibiting the initial actin aggregate phase, knockdown of cortactin using siRNA suppressed the subsequent sealing zone maturation process.

Osteoclast ability to attach to bone surfaces is essential for successful bone resorption. An intracellular calcium channel, inositol-1,4,5-triphosphate receptor-1 (IP3R1) is involved in the regulation of reversible osteoclast attachment.[198] IP3R1 can bind an endosomal

isoform of IP3R-associated cGMP-dependent kinase substrate (IRAG). Using siRNA against IRAG in human osteoclasts in vitro reduced cell spreading diameters and displayed distributed podosomes.[199] Loss of podosome ring structures explained reduced cell adhesion. Knockdown of the orphan nuclear receptor  $ERR\alpha$  in RAW 264.7 cells[200] generated similar effects as IRAG. Expression of osteopontin and the  $\beta 3$  integrin subunit is down-regulated after silencing  $ERR\alpha$ . Transfected cells detach from the substrate easily and no podosome belts are observed. Though cell adhesion and migration is impaired, their differentiation and precursor cell proliferation are not influenced by  $ERR\alpha$  knockdown.

Given the essential role of actin assembly in osteoclastic bone resorption, the motor protein family of myosins responsible for actin-based motility was examined in osteoclasts.[201, 202] Myosin X (Myo10) maintains low expression levels in most vertebrate tissues,[203] binding actin and microtubules.[204] Assembly of podosomes and sealing zones in osteoclasts depends on a complete intact microtubule system.[205, 206] RNAi-mediated inhibition of Myo10 in both RAW264.7 and mouse bone marrow-derived osteoclasts reduced the sizes of sealing zones and cell perimeters, but did not influence cell fusion.[202]

Brain-type creatine kinase (Ckb) exhibits high up-regulation upon osteoclast maturation as witnessed with proteomic technology.[207] Increased expression occurs in both mice bone marrow cells and RAW264.7 cells exposed to RANKL and M-CSF. Ckb regulates ATP distribution and supply in cells, required in osteoclast actin formation and maintenance.[208, 209] Down-regulation of Ckb in cell cultures produced prominent reduction in areas of resorption pits on dentin slices.[207] Reduced resorption ability was suggested to be partially due to impaired actin ring structures observed in transfected osteoclasts. In addition, Ckb was also shown to affect V-ATPase activity,[207] a major proton pump essential for osteoclastic resorption. Normal osteoclasts can recover their intracellular pH under challenge with strong acid over about 400 seconds. However, the lack of such intercellular pH recovery observed when inhibiting Ckb, infers that Ckb influences osteoclast bone resorption by affecting V-ATPase. In vitro cell culture data were confirmed in vivo through reduced bone surface erosion in ovariectomized  $Ckb^{-/-}$  mice. Interestingly, no significant differences in numbers of osteoclasts were observed between  $Ckb^{-/-}$  and wildtype mice.[207] Therefore, Ckb is a target for altering osteoclast activity, not osteoclastogenesis.

### 5.3. Osteoclast viability targets

In addition to targeting osteoclastogenesis and osteoclastic bone resorption, targeted induction of osteoclast apoptosis is also an efficient strategy to suppress excessive bone loss. EGFR is a target for regulating osteoclast differentiation (mentioned in Section 4.1). Inhibition of EGFR expression was also reported to cause apoptosis of mature osteoclasts through a caspase-9/caspase-3-dependent pathway by inhibiting the activation of P13K-Akt/PKB.[130] Taken together, siRNA against EGFR can not only inhibit osteoclast differentiation, but also suppress mature osteoclast survival.

RANK siRNA can be similarly characterized, as RANKL/RANK interaction is responsible for osteoclast differentiation, bone resorption and mature osteoclast survival as well. Therefore, mature osteoclasts transfected with RANK siRNA resulted in cell death.[67] Taken together, transfection of either osteoclast precursors or mature osteoclasts with RANK siRNA can reduce osteoclast-mediated bone resorption by inhibiting osteoclast differentiation, resorption activity and osteoclast survival.

Bisphosphonates, the most commonly used pharmacological approach for targeting osteoporosis currently, have several side effects which have clouded their therapeutic efficacy.[9, 10, 15] As an alternative, siRNA against alendronate's (a nitrogen-containing

bisphosphonate, N-BP) known molecular target,[210] farnesyl pyrophosphate synthase (FPPS), was investigated to inhibit osteoclasts and promote osteoblast activity simultaneously. FPPS plays an important role in the mevalonate pathway which produces lipids essential for cell survival.[211] Additionally, N-BPs are reported to induce human osteoblast differentiation and mineralization in culture by inhibiting the mevalonate pathway.[212] siRNA targeting FPPS significantly suppressed osteoclast cell viability with a single transfection and significantly increased osteoblast differentiation with effects sustained 5 days post-transfection in osteoblasts. However, this treatment does not significantly change osteoblast proliferation and mineralization compared to controls.[213] Therefore, selective knock-down of FPPS expression has the potential to inhibit osteoclasts while at the same time promoting osteoblastic activity.

Other siRNA targets described above influence osteoclast survival as well. NHA-oc/NHA2, mentioned as a target for osteoclastogenesis, inhibits osteoclast resorption activity partially because reduced NHA-oc/NHA2 express induces apoptosis by loss of inhibition of caspase-9 activation;[102] significant osteoclast death can also be induced by OGR1 siRNA transfection (section 4.1); siRNA against structurally unique osteoclast-specific transmembrane PTP-oc induces significant apoptosis in RAW/C4 osteoclast-like precursor cells. (section 4.2). In addition, many other general cell apoptosis signals are reported. Using siRNA targeting these proteins regulating these pathways, cell-specific delivery is essential to avoid undesired side effects.

#### 5.4. Other potential osteoporosis targets

Recognition of bone by osteoclasts is regulated by cell integrin receptors.[214] Integrins, as calcium-dependent, heterodimeric transmembrane protein receptors, mediate cell attachment with extracellular matrix or with other cells. Four different integrins are actively expressed in osteoclasts.[215] Among them,  $\alpha v\beta 3$  is highly expressed in osteoclasts and exhibits an important role in facilitating osteoclast attachment to bone and subsequent bone resorptive processes.[216] Osteoclast-mediated bone resorption was shown to be significantly inhibited by anti- $\alpha v\beta 3$  antibody treatment in vitro [217] and increased skeletal mass, absence of actin rings and abnormal osteoclast ruffled membranes were all observed in osteoclasts in  $\alpha v\beta 3$ -deficient mice.[216] Integrin  $\alpha v\beta 3$  recognizes the extracellular RGD peptide sequence and therefore RGD peptide has been used to label and target  $\alpha v\beta 3$ -positive cells in vivo.[218] In addition, inhibition of  $\alpha v\beta 3$  using a soluble RGD mimic as a receptor antagonist reduced osteoclastic bone resorption both in vitro and in vivo, suggesting that  $\alpha v\beta 3$  blockade prevents rapid bone loss caused by estrogen withdrawal.[219] In a study seeking to identify human peripheral blood monocyte subsets differentiating into osteoclasts, integrin- $\beta 3$  subunit siRNA was transfected into human CD16<sup>+</sup> monocytes.[220] This transfection significantly reduced TRAP-positive multinucleated cells in a dose-dependent manner. Silencing the  $\beta 3$  subunit in myeloma cells from human patients suppresses bone resorption activity (i.e., osteoclast-like activity) of these cells in vitro.[221] These data reflect the importance of  $\beta 3$  integrin subunit in RANKL-induced osteoclastogenesis. Therefore,  $\alpha v\beta 3$  could be an attractive target to attenuate or modulate osteoclastic bone resorption.

The soluble cell signaling protein, p53, is well known to protect cells from malignant tumor transformation by coordinating several intracellular signaling networks.[222] The ubiquitin ligase, murine double minute 2 (MDM2), regulates p53 activation by controlling p53 half-life.[223] Recently, a small molecule inhibitor, Nutlin, is reported to competitively bind MDM2 to interrupt p53-MDM2 interaction and activate the p53 pathway.[224, 225] In order to investigate the effect of Nutlin-3 on pre-osteoclastic precursor cell survival, proliferation and differentiation, human peripheral blood mononuclear cell pre-osteoclasts were transfected with p53 siRNA.[226] Results showed that Nutlin-3 anti-osteoclastogenesis activity was greatly compromised by p53 siRNA, indicating that the inhibitory effects of

Nutlin-3 on osteoclast precursors require the p53 activation pathway. Using siRNA technology to interrupt p53-MDM2 interactions may therefore have therapeutic implications for controlling excessive osteoclastic activity.

OGR1 was mentioned (*vida supra*) as a possible target for controlling osteoclastogenesis. This seven-pass transmembrane protein binds protons and exhibits pH-sensing activity.[109] Given that increased extracellular proton concentrations led to nuclear translocation of NFATc1, a downstream mediator of RANKL stimulation in osteoclasts,[227] OGR1's role, while still unclear, could be linked to bone pH homeostasis working as a proton sensor in response to external acidosis.[109] Therefore, further studies investigating OGR1 function in bone resorption sites is necessary to validate OGR1 as a target for osteoclast-mediated excessive bone resorption.

FPPS has been used as an siRNA target to inhibit osteoclast viability, mimicking the pharmacology of N-BPs (*vida supra*). However, depletion of intracellular geranylgeranyl pyrophosphate (GGPP) has also been reported to produce analogous phenotypic effects of bisphosphonate treatment on both osteoclasts and osteoblasts.[228, 229] Therefore, siRNA inhibition of GGPP synthase may induce apoptosis in osteoclasts while simultaneously promoting osteoblastic activities. Using siRNA against GGPP synthase could also potentially suppress excessive bone loss by stimulating osteoblastic bone production.

As osteoclasts form from cell fusion, cell-to-cell contact is critical for osteoclastogenesis. Cell adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1), whose expression is regulated by intracellular signalling through NF- $\kappa$ B and JNK, is involved in cell-cell contact between osteoclast precursors and osteoblasts/stromal cells, or among osteoclast precursors.[230, 231] ICAM-1 binds to integrin pairs, LFA-1 (CD11a/CD18), blocking interactions between ICAM-1 and LFA-1, inhibiting osteoclast formation.[230] Hidetaka et al. reported that siRNA knock-down of LFA-1 in RAW264.7 cells had no effects on osteoclastogenesis. Alternatively, ICAM-1 could be an siRNA target to inhibit osteoclasts as ICAM-1 is not only expressed on osteoclast precursors and mature osteoclasts, but also on osteoblasts.[232, 233] Therefore, cell-specific targeted delivery will be essential for the specific bioactivity and siRNA selectivity to osteoclasts as desired.

## 6. siRNA delivery to bone

### 6.1. Challenges and strategies

RNAi as a novel therapeutic approach has considerable potential to silence abnormal genes, especially for gene targets not effectively targeted by conventional therapeutics (*i.e.*, by small molecules, proteins and antibodies). The major obstacle for developing siRNA for therapeutics is also its targeted delivery with clinically acceptable formulations and reliable routes of administration. This is particularly true of siRNA delivery to bone with its intrinsically poor drug penetration and vascular perfusion. Bone is primarily composed of three cell types: osteoclasts, osteoblasts, and osteocytes. Bone's unique extracellular matrix is strongly mineralized with calcium phosphate, forming ~70 wt% apatite, a major reservoir of the body's calcium most actively involved with physiological calcium and phosphorus homeostasis. Increasing molecular and cellular understanding of bone biology has produced continuous reports of new potential therapeutic targets for various bone pathologies. Nonetheless, despite new targets identified as osteoclast-specific, targeting methods are not yet reliably tissue-based specifically with requisite bone-specificity necessary for these therapeutic agents. Serious complications from cross-reactivity with other non-target tissues or poor efficacy due to low drug target tissue concentration require a reliable targeting strategy. Many attempts to improve the targeting of drugs to bone mineral fraction (apatite) include drug conjugation with bone-targeting ligands, such as tetracycline,[234] estradiol,

and bisphosphonate.[235, 236] Bisphosphonates, especially N-BPs, as anti-resorptive drugs having intrinsic high affinity to the bone apatite surface, are very attractive for delivery of conjugated non-specific bone therapeutic agents. However, their exploitation as bone-targeting agents must consider that free bisphosphonate itself also will increase anti-resorptive activity.

siRNAs are well known for their very short circulating half-lives in vivo.[237] Unmodified naked siRNA cannot be directly administered directly in vivo, exhibiting only a 6 min half-life after systemic administration to rats due to degradation by serum nucleases.[237] Many studies seek to overcome the challenges of siRNA delivery by the chemical modification of siRNA nucleotides, lipid/liposomal/polymer-based complexes, and collagen (atelocollagen)-based[238, 239] formulations for siRNA, as well as viral vectors and polymer-complexed siRNA carriers.[240] These methods each have their advantages and disadvantages, but non-viral delivery is considered a more clinically relevant delivery mechanism, avoiding the known problems of current viral vector delivery: high viral toxicity, possible carcinogenicity, proven immunogenicity, and significant cost limitations.[31] Delivery of siRNA targeted specifically to bone is not an entirely new story. To date, in vivo siRNA studies in humans have focused on rheumatoid arthritis (RA) by local delivery (intra-articular injection[241, 242], electroporated siRNA[243], topical cream[34]), or by systemic delivery, each with modest effects.[244] Delivery of siRNA to bone osteoclasts in vivo, either targeted to or addressing, is not yet reported.

To improve siRNA stability in vivo, various chemical modifications have been investigated. siRNA stability against nuclease degradation can be improved by introducing a phosphorothioate (P=S) backbone linkage or modifying the ribose 2'-hydroxyl position as 2'-OMe or 2'-F, as well as a 4'-thioribose modification.[245] All show significant improvements over unmodified siRNA.[246–248] Combination of 2'- and 4'-thioribose modification results in substantially improved siRNA bioactivity and plasma stability.[249] This improved siRNA plasma stability facilitates more reliable systemic dosing and versatile delivery options. In addition, specific siRNA chemical modifications can potentially be used to improve conjugation and targeting as well. Bone-targeting ligands, antibodies[250] or small molecule drugs (vida supra) can be conjugated with siRNA molecules with various chemistries for systemic delivery of siRNA-based bone disease therapeutics. For example, bisphosphonates, can be conjugated to ribose hydroxyl groups in siRNA sugars. Modification of the ribose 2'-OH at the 3' end of the guide siRNA strand appears effective for improving siRNA activity.[240] In this case, bisphosphonate performs both as a drug and also as a targeting moiety to deliver siRNA specifically to bone.

Using tissue-specific targeting methods, both locally and systemically delivered siRNA can be more reliably dosed by judicious combination of other “homing” molecules. The concept of siRNA targeting to improve both systemic and local efficacy is shown in Figure 4. Established drug delivery strategies can be utilized for both targeted systemic and local siRNA delivery, including siRNA conjugation to cell membrane-targeting ligands, antibodies, water-soluble polymers,[251] or cell-penetrating peptides. These methods seek to improve amount of siRNA delivered specifically to targeted bone cells, and to limit drug off-target effects in cells other than osteoclasts.

RANK expressed on osteoclast precursors as well as on mature osteoclasts serves as a popular molecular target for osteoporosis therapies. Antibodies against RANK have been tested for effective osteoclast targeting by conjugation with calcitonin, an anti-resorptive protein drug.[252] The conjugate showed efficacy inhibiting osteoclast formation and bone resorption. The strategy, using receptor ligand binding peptides as the targeting moiety, provides a general approach to generating an osteoclast-targeting platform for specific drug



delivery to osteoclasts. Other osteoclast-specific proteins can also be utilized for this purpose, such as cathepsin K and NHA-oc/NHA2. In addition, many calcium-binding proteins, recently reviewed,[253] including acidic proline-rich salivary proteins, osteocalcin, sialoprotein, and osteopontin, have the potential as novel bone-targeting moieties.[253] The hexapeptide (Asp)<sub>6</sub> exhibited high affinity to bone and was used as bone-targeting moiety conjugated with estradiol in ovariectomized mice. The conjugates maintained bone density similarly to unconjugated estradiol, but without displaying increased liver and uterus weight. [254, 255]

In addition to specific chemical modifications, protection of siRNA in plasma is also provided by formulating siRNA into nano/macro-sized particles for delivery, as recently reviewed.[256–258] Most drug delivery strategies to date primarily target the liver, either deliberately or non-discriminately, a natural result of first-pass metabolism and highly efficient liver-based drug and colloidal clearance. This is an advantage for liver targeted therapies: for example, hydrodynamic tail-vein injection of Fas siRNA successfully protected mice from liver failure and fibrosis.[259] However, the liver and other reticuloendothelial (MPS/RES) system tissues efficiently filter most drug carrier particles (i.e., >90%) despite active targeting to other target tissue sites. To avoid this massive dose removal, locally delivered siRNA-particle formulations can be used in bone-targeted therapeutics. Specifically to target osteoclast or osteoclast precursors, siRNA delivery can exploit drug carrier particle size-selective cell-specific uptake to numerous phagocyte target cells in bone tissue using local delivery. In the context of osteoporosis therapy, targeted cells are pre-osteoclastic monocytes and osteoclasts -- all naturally phagocytic cells. Phagocytes efficiently take up particles with diameters up to 10  $\mu\text{m}$ . [260] However, particles with diameters greater than 300 nm are too large to penetrate non-phagocytic cell membranes (i.e., osteoblasts, fibroblasts) either passively or by endocytosis or pinocytosis.[261] Therefore, siRNA-loaded microparticles with controlled sizes could be exploited for selective internalization by osteoclastic phagocytic mechanisms, and to target siRNA to these cells in new osteoporotic therapeutic strategies without a conjugated targeting moiety. These particles could be delivered locally to bone sites in clinical cases, especially in bone augmentation and implant surgeries. Additionally, for some bone molecular drug targets appropriate both for osteoclasts and osteoblasts, siRNA-containing nanoparticles capable of being taken up by both cell types might be effective for increasing bone mass by inhibiting osteoclasts while simultaneously stimulating osteoblasts. Particle opsonisation by host plasma proteins, commonly a problem for drug carriers, may confound target cell phagocytosis since opsonisation (particularly, non-specific adsorption of immunoglobulins and complement onto the particles) may activate macrophages and T-cells and cause inflammation in surrounding tissues and damage to healthy cells.[262]

Currently, local siRNA administration is more efficient than its systemic delivery for short-term therapeutic purposes.[263] Nonetheless, local delivery must also consider target specificity. For example, intra-articular injection of siRNA for RA is not an effective route of administration for transfecting osteoclasts located near or on bone surfaces at most common osteoporotic bone sites, notably pelvis, vertebrae and wrist.[264] To maximize drug availability to specific bone sites and obtain a reliable, reproducible and predictable pharmacokinetic profiles, bioactive materials, including porous chitosan/collagen scaffolds[265] and injectable FDA-approved calcium phosphate bone cements (CPC) are being developed as drug carriers for local bone delivery. Polymethylmethacrylate (PMMA) bone cements used clinically for implant fixation, kyphoplasty,[266] and bone augmentation,[267] exhibit very poor control over entrapped dose release kinetics, often leaving up to 40% of the drug trapped in PMMA.[268, 269] Injectable CPC exhibits several unique properties for bone drug delivery, including peri-operative preparation and drug loading, liquid-solid setting in situ, intrinsic osteoconductivity (degradable by osteoclasts),

osteoinductivity (infiltrated by osteoblasts and replaced with new bone), and acceptable biocompatibility.[270–272] CPC's self-setting ability at ambient or body temperature within the bone cavity [273] enables injectable formulation, largely expanding its therapeutic utility.[274, 275] To date, a large body of evidence supports the feasibility and value of using CPC as a local drug carrier in bone augmentation. Pharmacologically active molecules are readily dispersed or distributed throughout CPC prior to setting and surgical placement, providing a sustainable and controlled drug release medium. Several conventional small molecule and protein-based drugs have been delivered using CPC in bone, including antibiotics to decrease post-surgical infections,[276–279] anticancer drugs to reduce tumorigenesis[280, 281] and growth factors to promote bone healing.[282, 283] Therefore, despite its limited mechanical properties, CPC could also be used to deliver siRNA to bone as a local delivery matrix.

A degradable cationic hydrogel comprising of gelatin and chitosan has been used for local delivery of an antisense oligonucleotide targeting murine TNF- $\alpha$  to suppress endotoxin-induced osteolysis in mouse calvaria.[284] Cationic polymeric chitosan complexes with anionic nucleotides and also facilitates cell transfection. Osteoclast numbers and bone resorption were significantly suppressed 4 weeks post-implantation. The same idea can also be extended to siRNA local delivery to bone.

The intrinsically low pH in osteoclast resorption lacuna and in the cell lysosome is attractive as a basis for targeting drugs conjugated to carriers containing acid-cleavable hydrazone bonds.[285] Additionally, various acid-sensitive, cathepsin K-specific and MMPs-specific peptide linkages (reviewed recently[253]) also provide disease- and environmentally specific bone-targeting strategies appropriate for targeted siRNA bone delivery.

## 6.2. Non-specific siRNA-cell bioactivities and off-target side effects

The first clinical trials involving siRNA were applied by local intraocular injection in patients with choroidal neovascularization (CNV).[33, 286] CNV is an age-related eye disorder characterized by the choroidal vessel invasion to the retina beneath retinal pigmented epithelium. Naked siRNA targeting VEGFA or VEGFR1 showed significant suppression in a laser-injury-induced CNV mice model.[33, 286] However, in 2008, new data showed that this therapeutic suppression is a general siRNA-class effect, independent of siRNA sequence.[25] The observed therapy was based on interactions between cell-surface (but not endosomal) toll-like receptor 3 (TLR3), a cell membrane double-stranded viral RNA sensor found on numerous cell types,[287] and siRNA, regardless of RNA sequences, target, and internalization. However, to bind TLR3, siRNA of 21 nucleotides or longer was required to form a 2:1 TLR3-RNA conjugate. TLR3 plays an indispensable role in the TRIF--NF- $\kappa$ B cell signalling cascade where TRIF is the TLR3 adaptor protein.[288] This important discovery has revealed generic properties of siRNAs on vascular tissues, and improved the global understanding of siRNA general bioactivities, both desired and potentially undesired, through non-specific signalling pathways.

In this regard, primary human choroidal endothelial cells showed decreased survival in serum-containing media cultured with serum-stable 2'-O-methyl-Lus-siRNA (without transfection reagents).[25] Such extracellular siRNA-induced cytotoxicity might affect other organs, including bone. Osteoclast precursors but not mature osteoclasts express TLR3.[59] Poly(I:C)dsRNA, which acts as a TLR3 ligand to stimulate cell surface TLR3, produced strong inhibition of osteoclast differentiation in both mouse bone marrow cultures and human peripheral blood monocytes at 1  $\mu$ g/ml in a dose-dependent manner under induction by M-CSF and RANKL. TLR stimulation induces the production of various proinflammatory cytokines such as TNF- $\alpha$ [289] and prompts high levels of phagocytic activity in osteoclast precursors,[59] indicating an enhanced immune response after TLR

stimulation in osteoclast precursors. Taken together, both the unanticipated vascular and immune-general siRNA-class effects should be carefully considered for siRNA therapies, especially when delivered systemically.

## 7. Conclusions

Gene silencing using siRNA has many potential therapeutic applications due to several advantages intrinsic to RNAi, such as its high target specificity and intrinsic biological response.[28] Effective siRNA delivery and selective targeting to desired tissue sites remains problematic. siRNA delivery to bone has developed relatively slowly compared to its delivery to other tissues. In this regard, osteoporosis is an increasingly challenging problem globally with a need for improved therapeutics. Many new cellular targets in osteoclasts are continually reported and attractive for siRNA knock-down. Efficient siRNA delivery methods and selective targeting to bone connective tissue and osteoclasts must be developed, improved and optimized. siRNA's efficacy, bioavailability, and therapeutic duration in the context of osteoclast-mediated excessive bone resorption are largely unexplored, but this strategy has attractive options for development of new therapeutics.

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## Abbreviations

<b>3BP2</b>	c-Abl SH3 domain-binding protein-2
<b>ADAM</b>	a disintegrin and metalloproteinases
<b>Ago</b>	Argonaute
<b>AP-1</b>	activator protein-1
<b>Ckb</b>	brain-type creatine kinase
<b>CNV</b>	choroidal neovascularization
<b>CPA</b>	cation-proton antiporter
<b>CPC</b>	calcium phosphate bone cements
<b>DC-STAMP</b>	dendritic cell-specific transmembrane protein
<b>EGFR</b>	epidermal growth factor receptor
<b>FPPS</b>	farnesyl pyrophosphate synthase
<b>GGPP</b>	geranylgeranyl pyrophosphate
<b>H<sup>+</sup>-ATPase</b>	H <sup>+</sup> -adenosine triphosphatase
<b>ICAM-1</b>	intercellular adhesion molecule-1
<b>IKK</b>	I $\kappa$ B kinase
<b>IP3R1</b>	inositol-1,4,5-triphosphate receptor-1
<b>IP3R1</b>	inositol-1,4,5-triphosphate receptor-1
<b>KCCs</b>	K <sup>+</sup> /Cl <sup>-</sup> co-transporters
<b>M-CSF</b>	macrophage colony-stimulating factor

<b>MDM2</b>	murine double minute 2
<b>N-BP</b>	nitrogen-containing bisphosphonate
<b>NCX</b>	Na <sup>+</sup> /Ca <sup>2+</sup> ion exchanger
<b>NFATc1</b>	nuclear factor of activated T cells
<b>NF-κB</b>	nuclear factor kappa B
<b>OCIF</b>	osteoclastogenesis inhibitory factor
<b>OC-STAMP</b>	osteoclast stimulatory transmembrane protein
<b>OGR1</b>	ovarian cancer G-protein-coupled receptor 1
<b>OPG</b>	osteoprotegerin
<b>PBMC</b>	blood mononuclear cells
<b>PIP<sub>2</sub></b>	phosphatidylinositol 4,5-bisphosphate
<b>PTK</b>	protein tyrosine kinase
<b>PTP-oc</b>	osteoclast-specific transmembrane protein-tyrosine phosphatase
<b>PTP-PEST</b>	protein-tyrosine phosphatase-proline, glutamic acid, serine, threonine amino acid sequence
<b>RA</b>	rheumatoid arthritis
<b>RANK</b>	receptor activator of NF-κB
<b>RANKL</b>	RANK ligand
<b>RGS</b>	regulators of G-protein signalling
<b>RISC</b>	RNA-induced silencing complexes
<b>RIZ1</b>	retinoblastoma protein-interacting zinc finger 1
<b>RNAi</b>	RNA interference
<b>shRNA</b>	short hairpin RNA
<b>siRNA</b>	small interfering RNA
<b>TLR3</b>	toll-like receptor 3
<b>TRAF6</b>	TNF receptor-associated factor 6
<b>TRAIL</b>	TNF-related apoptosis-inducing ligand
<b>TRAP</b>	tartrate resistant acid phosphatase
<b>TREM2</b>	triggering receptor expressed in myeloid cells-1
<b>WASP</b>	Wiscott-Aldrich syndrome protein

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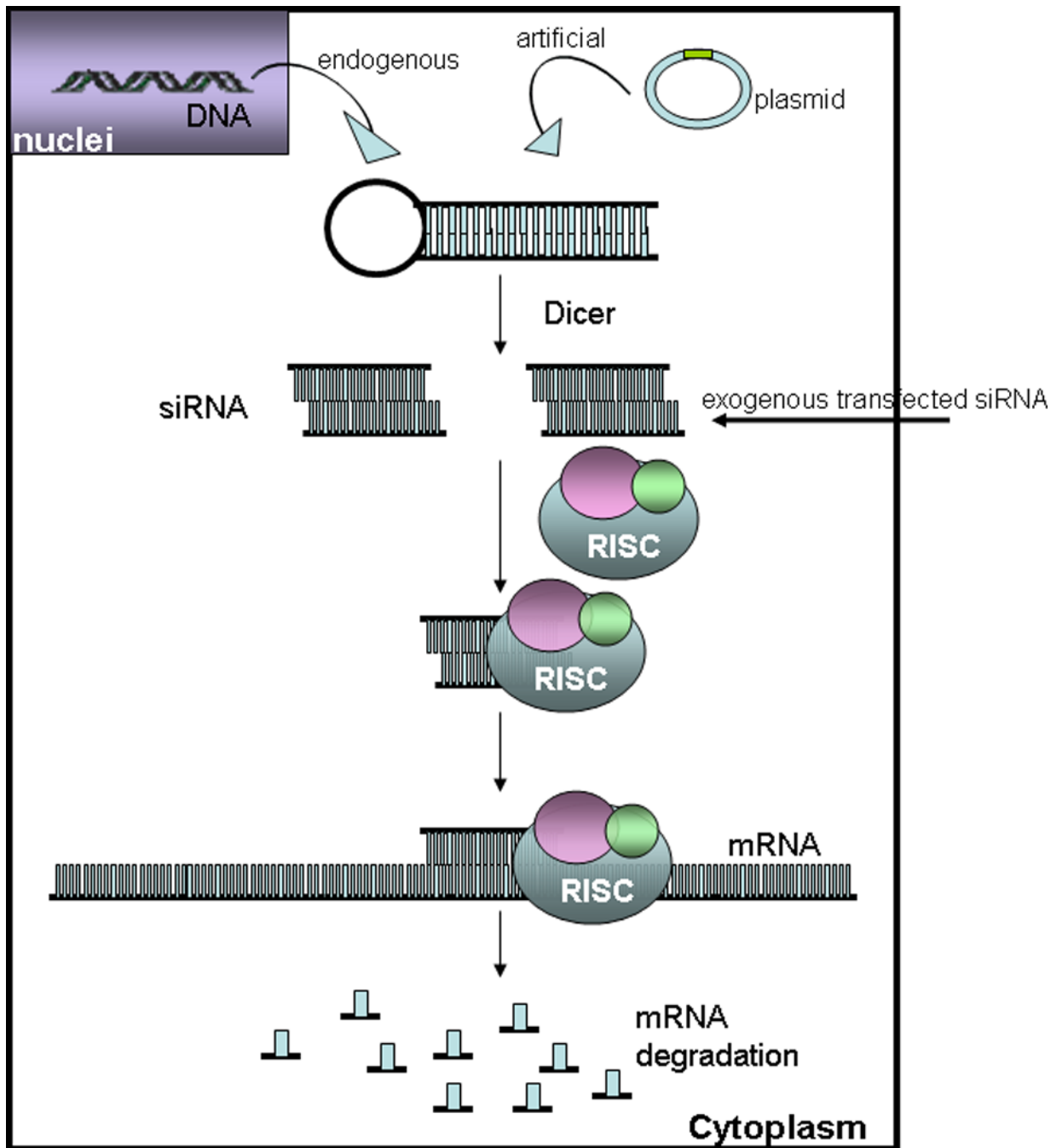


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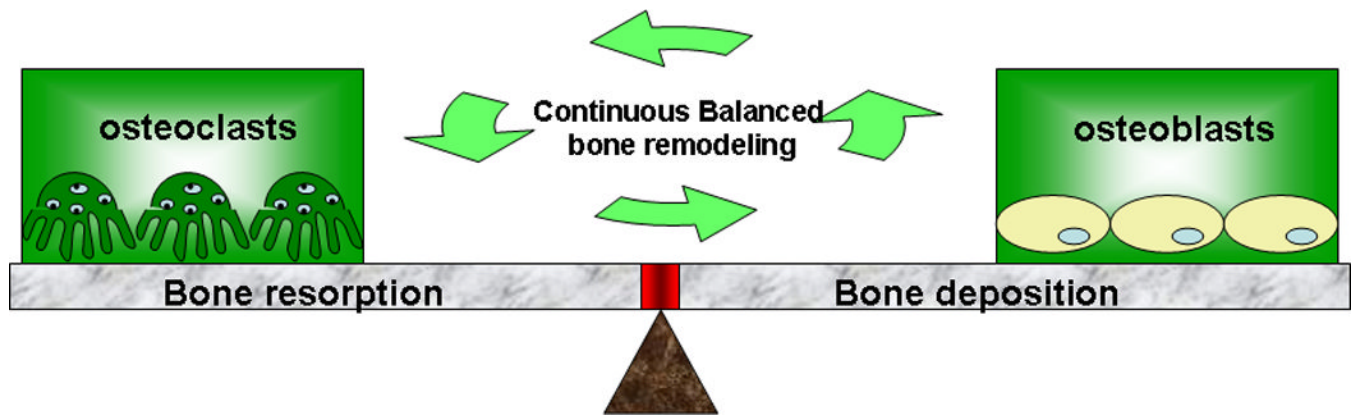
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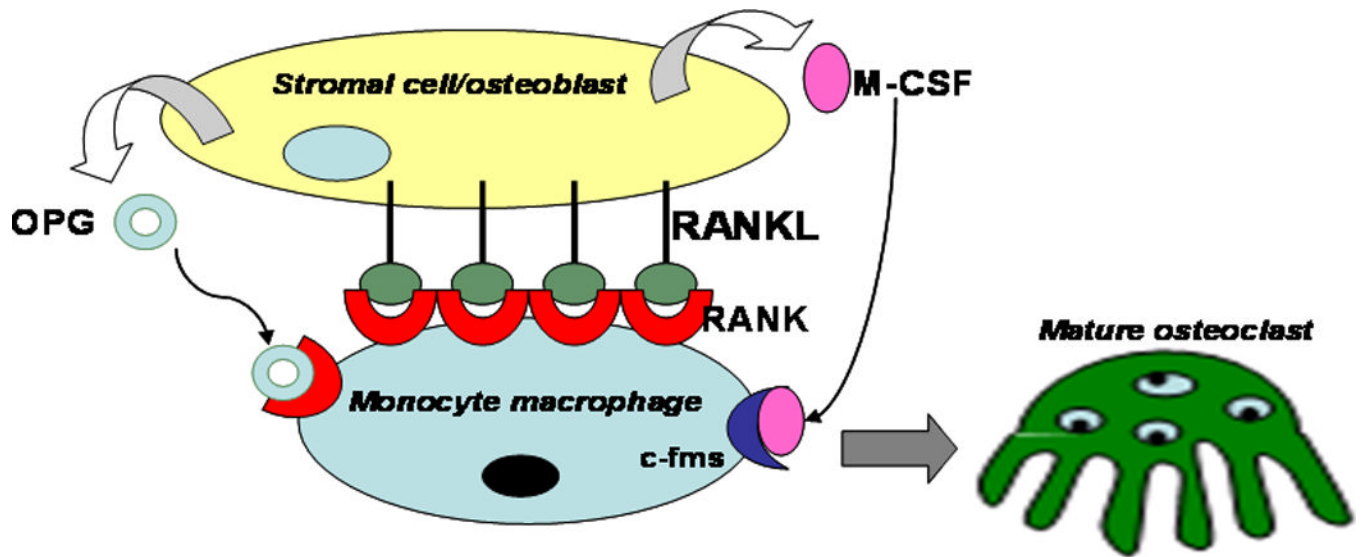
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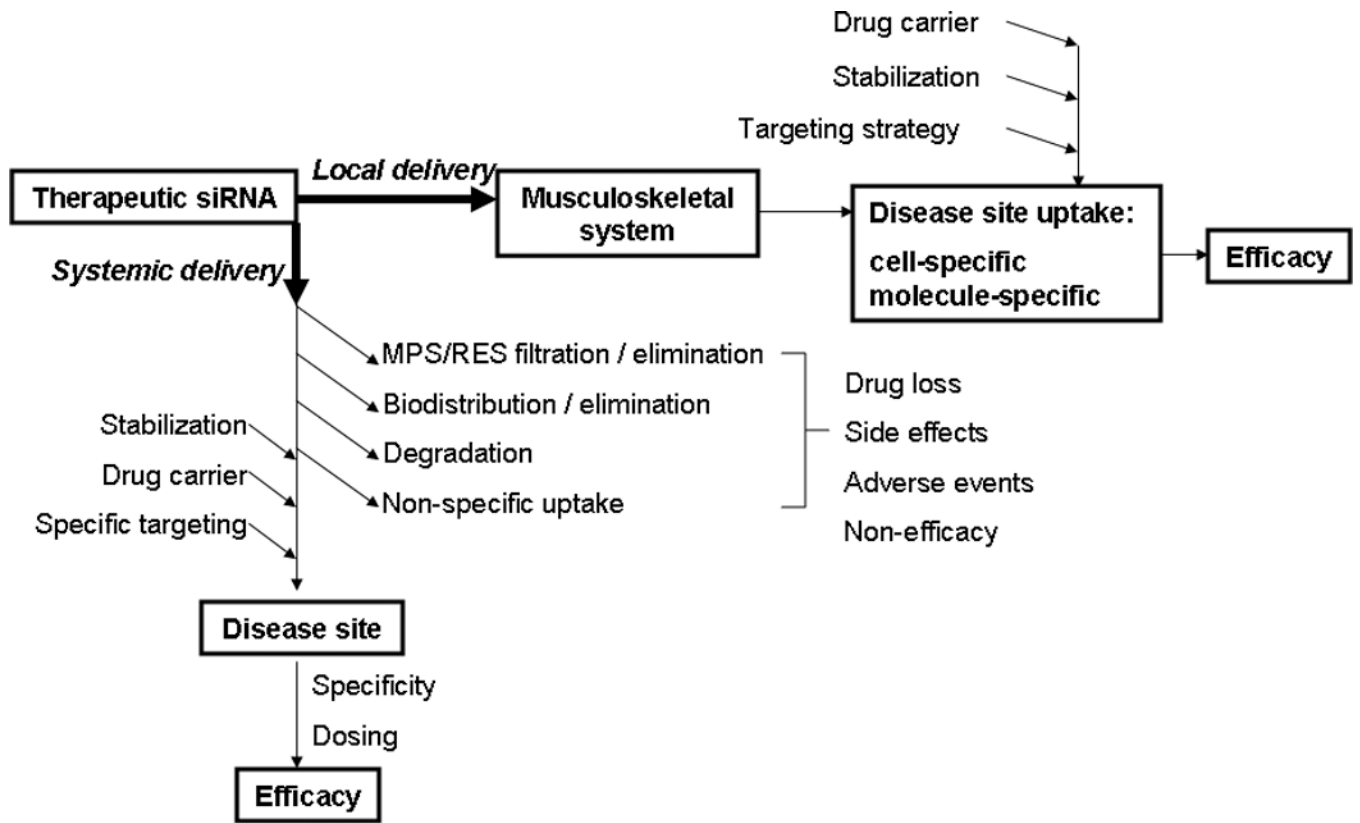
**Figure 1.**  
RNA interference mechanism.



**Figure 2.** Normal bone metabolic homeostasis as a balance of bone resorption and deposition mechanisms. This balance goes awry in osteoporosis pathology.



**Figure 3.** Cellular mechanisms of osteoclastogenesis involving osteoblast/macrophage precursors cell interactions, mediated by RANK, RANKL, and OPG among other signaling partners.



**Figure 4.** The concept of therapeutic siRNA targeting delivery to improve siRNA bioavailability and therapeutic efficacy.