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► **To cite this version:**

Pinpin Wang, Federico Perche, Delphine Logeart-Avramoglou, Chantal Pichon. RNA-based therapy for osteogenesis. *International Journal of Pharmaceutics*, 2019, 569, pp.118594. 10.1016/j.ijpharm.2019.118594 . hal-02995602

HAL Id: hal-02995602

<https://hal.science/hal-02995602v1>

Submitted on 24 Nov 2020

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RNA-based Therapy for Osteogenesis

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Abstract (less than 200 words)

Nucleic acid-based therapy has shown great promise in accelerating bone regeneration as well as other diseases. Nucleic acids used in gene therapy mainly are either plasmid DNA (pDNA) or RNAs. Although pDNA therapy has been extensively studied for decades with encouraging preclinical and clinical results, side effects and low efficiency associated with nuclear trafficking are hard to bypass. Unlike pDNA, RNAs (mRNA, siRNA, miRNA) exert their function in the cytoplasm thereby being more efficient in hard-to-transfect cells such as primary osteoblasts. RNA interference-based gene silencing represents a negative regulation which knockdown the expression of antagonists that impair osteogenesis process. In contrary, mRNA therapy for osteogenesis represents a positive regulation which delivers mRNA encoding growth factors to accelerate bone regeneration. This review presents a comprehensive summary of the mRNA and siRNA-based therapies and the targets for bone regeneration in case of bone defect and osteoporosis. Keywords: osteogenesis, gene therapy, RNA interference, mRNA, protein replacement, osteoporosis

Contents

1. Introduction

Bone tissue has a self-healing capacity which is overwhelmed during traumatic events and bone pathologies. A constant homeostatic balance between bone formation by osteoblasts and bone resorption by osteoclasts is essential for bone tissue to restore fatigue microdamage and maintain bone mass and structure (Rodan and Martin 2000). However, imbalances are associated with pathological or traumatic events. For instance, during some pathologies such as osteoporosis and osteonecrosis, osteoclasts are more active compared to osteoblasts resulting in low bone mass, poor bone strength, and micro-architectural deterioration of bone tissue ((Roush 2011); (Genant, Cooper et al. 1999)). This imbalance increases the risk of bone fracture and subsequently delays bone healing, even leading to nonunion fractures (5-10% of cases) [(Egermann, Schneider et al. 2005)]. The occurrence of critical-sized

defects following for instance either traumas or resection of osteosarcomas are additional challenges to the self-healing capacity of bone [(Dimitriou, Tsiridis et al. 2005)].

Osteoporosis- and bone fractures are becoming a major health and economic burden with a progressively aging population. In the European Union, the estimated osteoporotic population was 27.5 million (22 million women and 5.5 million men) in 2010. The cost of osteoporosis-related incidents and osteoporosis-related fractures was estimated at 37 billion Euro in 2010, which is predicted to increase by 25% in 2025 (Svedbom, Hernlund et al. 2013).

Clinically, to reverse the loss of bone mass, there are two major pharmacological approaches based on either: 1) anabolic agents such as parathyroid hormone (PTH) to stimulate bone formation; or 2) antiresorptive agents such as bisphosphonate, estrogens, and selective estrogen receptor modulators to prevent bone resorption (NIH Consensus Development Panel on Osteoporosis Prevention and Therapy 2001). However long-term administration of anabolic agents or antiresorptive agents is accompanied by adverse effects. For example, bisphosphonate-based therapy has been associated with jaw osteonecrosis and atypical fractures in long bone [(Schilcher, Michaelsson et al. 2011); (Hollick and Reid 2011)]. To restore bone defects, although the success rate has been significantly improved with the development of sophisticated surgical procedures, e.g. Ilizarov apparatus [(Robert Rozbruch, Weitzman et al. 2006)], Masquelet technique [(Giannoudis, Faour et al. 2011)], autografts still represent the gold standard treatment to treat massive bone loss [(Goldberg and Stevenson 1987); (Giannoudis, Faour et al. 2011)].

This last decade, it is admitted that there is a need to identify novel therapeutic strategies to enhance bone formation without side effects. Romosozumab, a monoclonal antibody targetting sclerostin, was developed as an anabolic and antiresorption drug to treat osteoporosis (Padhi, Jang et al. 2011); (McClung, Grauer et al. 2014); (Cosman, Crittenden et al. 2016). Sclerostin is a negative regulator of bone formation by inhibiting Wnt/ β -catenin pathway and positive regulator of bone resorption by activating RANK/RANKL pathway (Suen and Qin 2016). However, despite promising results in a recently finished phase three trial, some serious adverse events have been reported such as hyperostosis and cardiovascular events (Cosman, Crittenden et al. 2016).

To repair bone defect, bone tissue engineering represents an interesting therapeutic alternative to bone grafts. Bioengineered constructs containing a biocompatible scaffold combined with osteogenic cells and/or recombinant osteogenic proteins are increasingly being used as bone graft substitutes [(Amini, Laurencin et al. 2012)]. Although promising results showed bone reparation in clinical studies, many crucial hurdles remain such as side effects related to the supraphysiological dose of implanted osteogenic proteins [(Shimer, Oner et al. 2009)].

Gene therapy has been proposed for bone regeneration many years ago. The goal of this approach has been to either sustain or modulate the expression of therapeutic proteins. In vivo and ex vivo strategies have been performed mainly using viral vectors. The pDNA-based gene therapy for osteogenesis will not be covered in the present review, as it has been the focus of recent excellent published reviews [(Evans and Huard 2015);(Im 2013)].

Combining RNAs encoding osteogenic factors, complexed within vectors, in scaffolds in the presence or absence of osteogenic cells is among the most advanced gene therapy ?? solutions for bone healing. The scaffolds can be loaded with either messenger RNA (mRNA), mimics of endogenous microRNA (miRNA), or siRNA to deplete negative regulators of bone formation (Elangovan, Khorsand et al. 2015); (Khorsand, Elangovan et al. 2017); (Takayama, Suzuki et al. 2009). In addition to local release from the scaffold, RNAs can be routed to the bone damaged area using a targeted delivery system, Additional to the local release, RNAs can be systemically administered to target the whole skeleton (Zhang, Wei et al. 2015); (Sun, Ye et al. 2016). In this review, we will discuss the progress of mRNA and siRNA-based gene therapy for the treatment of either bone defects or loss bone mass. But to appreciate the goals of the various strategies, it is needed to give insights on how the bone homeostasis is maintained and what types of molecules are involved during bone regeneration.

2. Signaling pathways involved in bone homeostasis

Osteoblasts and osteoclasts are the two major cells involved in bone remodeling or bone defect-healing process. Their recruitment, proliferation, differentiation, activity and survival are tightly regulated by dozens of autocrine and paracrine factors through multiple signaling pathways such as Bone Morphogenetic Proteins (BMPs)/ runt-related transcription factor 2 (Runx2) (Hankenson, Gagne et al. 2015); (Phimphilai, Zhao et al. 2006), Wnt/ β -catenin (Fischer, Boland et al. 2002); (Rawadi, Vayssiere et al. 2003), and receptor activator of nucleic factor- κ B (RANK)/ RANK ligand (RANKL) pathways (Wada, Nakashima et al. 2006); (Zhuang, Yoshizawa-Smith et al. 2018). [Fig. 1.]

Bone formation is generated by osteoblasts that promote bone matrix deposition and mineralization. Osteoblasts originate from mesenchymal stem cells (MSCs) that commit and differentiate under the control of various stimuli that influence osteogenesis. One of the key molecular switches in the commitment of mesenchymal progenitors to osteoblast lineage is the transcription factor *cbfa/runx2*, which has multiple upstream regulators and a wide variety of targets¹. The Wnt and BMP signaling

¹ Doi: 10.12659/MSMBR.901142

pathways are the major regulatory pathways involved in the regulation of Runx2. BMPs are cytokine members of the TGF- β family that are especially important for postnatal ossification. Among them BMP2, 7 and 9 have been incorporated in bone graft substitutes as recombinant proteins or mRNA (refs). (Hankenson, Gagne et al. 2015). BMP signaling is initiated by the ligand binding to type I and type II BMP receptors which are serine/threonine kinase receptors complexing into transmembrane heterodimer. Upon binding of BMPs to their receptors, the activated receptors initiate two intracellular transduction pathways: the canonical Smad pathway and non-canonical mitogen-activated protein kinase (MAPK) pathway. In canonical pathway, activated receptor recruit and phosphorylate Smad 1/5/8, which are translocated to the nucleus associated with Smad 4. The Smads complex together with Runx2 activates the expression of downstream osteogenic genes such as alkaline phosphatase (ALP), osteocalcin (OCN), osteopontin (OPN) (Chen, Deng et al. 2012). The MAPK pathway is Smad independent and includes the activation of extracellular signal-regulated kinase 1/2 (ERK1/2), p38, and c-Jun NH2-terminal kinase (JNK1/2) resulting in Runx2 expression upregulation (Huang, Yuan et al. 2014).

The BMP/Smad signalling cascade is tightly regulated by both intracellular (such as Smurf proteins) and extracellular processes. A family of secreted antagonists that negatively regulated the BMP/Smad signaling pathway includes Noggin [(Smith and Harland 1992)], Gremlin [(Hsu, Economides et al. 1998); (Pearce, Penny et al. 1999)], Chordin [(Piccolo, Sasai et al. 1996)], crossveinless, USAG-1 and follistatin. Coexpressed with BMP-2, -4, and -7, the Noggin, Gremlin, and Chordin antagonists play key roles during development and skeletogenesis [(Zimmerman, De Jesus-Escobar et al. 1996); (Merino, Rodriguez-Leon et al. 1999); (Scott, Steiglitiz et al. 2000); (Fisher and Halpern 1999)]. After secretion, these antagonists physically sequester BMPs, preventing ligand-receptor interactions.

Wnt/ β -catenin signaling is another well-defined signaling pathway that promotes osteoblastic differentiation and activity. Wnt proteins constitute a family of secreted cysteine-rich glycosylated proteins. Activation of Wnt/ β -catenin signaling pathway initiates from binding of the Wnt ligands with the complex of the 7-transmembrane domain spanning frizzled receptor and low-density lipoprotein receptor-related protein 5 and 6 (LRP5/6) coreceptors. Activation of the Wnt receptor complex triggers displacement of the multifunctional kinase GSK-3 β from a regulatory APC/Axin/GSK-3 β -complex, resulting in β -catenin stabilization and accumulation. The β -catenin translocates to the nucleus regulating osteogenic genes expression (Krishnan, Bryant et al. 2006). Wnt signaling also reduces apoptosis of osteoblasts and osteocytes through mechanisms involvement of β -catenin and activation of PI3K/Akt (You, Saims et al. 2002); (Longo, Kennell et al. 2002).

Several antagonists are involved in Wnt/ β -catenin signaling regulation. Among them, Dickkopf families (DKK1/2/4) bind to LRP5/6 complex leading to the LRP internalization and degradation (Mao, Wu et al. 2002). Also, the interaction of Wnts and frizzled receptors is inhibited by frizzled-related protein family (sFRP1/2/3) which sequester Wnts (Krishnan, Bryant et al. 2006).

Bone resorption is mediated by osteoclasts which are giant multinucleated cells derived from monocytes/macrophages. Osteoclasts resorb the bone tissue by dissolution of the inorganic phase through acidification of the isolated extracellular microenvironment followed by secretion of proteolytic enzymes. The osteoclast differentiation is locally regulated by RANK/RANKL signaling under the presence of macrophage colony-stimulating factor (M-CSF) (Wada, Nakashima et al. 2006). RANKL, a TNF-family molecule, either expressed on the surface of osteoblastic lineages or secreted from stromal or immune cells, interacts with RANK receptor in osteoclastic lineages. The RANK signaling transduction pathway is regulated by the adaptor proteins of TNF receptor-associated factor 1, 2, 3, 5 and 6 (TRAF 1, 2, 3, 5, 6) within which TRAF 6 is critical for RANK signaling (Koide, Kinugawa et al. 2010). The TRAFs binds to RANK and transmits the RANK signal to downstream targets including nuclear factor-kappa B (NF- κ B) pathway and MAPK pathway resulting in osteoclastic differentiation of macrophages cells, activation of bone resorption, and survival of osteoclasts. For example, ERKs and JNKs stimulate transcription factor Activating Protein-1, a dimer composed of c-Fos and c-Jun protein, together with activated NF- κ B regulate the expression of nuclear factor of activated T cells c1 (NFATc1, also called NFAT2) leading to enhanced expression of osteoclastogenic genes. Proto-oncogene tyrosine-protein kinase Src (c-SRC) mediates RANK induced activation of the phosphatidylinositol 3-kinase (PI3K)/Akt signaling through binding to TRAF6 for anti-apoptosis (Nakashima, Kobayashi et al. 2000). Osteoblasts also secrete a RANKL decoy receptor-osteoprotegerin (OPG) that competitively binds RANKL and, thus, inhibits osteoclast differentiation and survival (Hofbauer and Heufelder 2001); (Maxhimer, Bradley et al. 2015).

RANKL induced-osteoclastogenesis stimulates semaphorn 4d (sema4d) expression from osteoclasts which, in turn, regulates osteoblasts activity (Negishi-Koga, Shinohara et al. 2011). Sema4d is originally expressed as a transmembrane protein then proteolytically cleaved into a soluble form (Suzuki, Kumanogoh et al. 2008). Binding of Sema4d to Plexin-B1, the Sema4d transmembrane receptor in osteoblast, inhibits Akt and Erk phosphorylation resulting in reduction of osteoblast mediated bone-formation.

The unraveling of the signaling pathways and identification of the regulators involved in bone formation and resorption enable to rationally design targeting therapeutics.

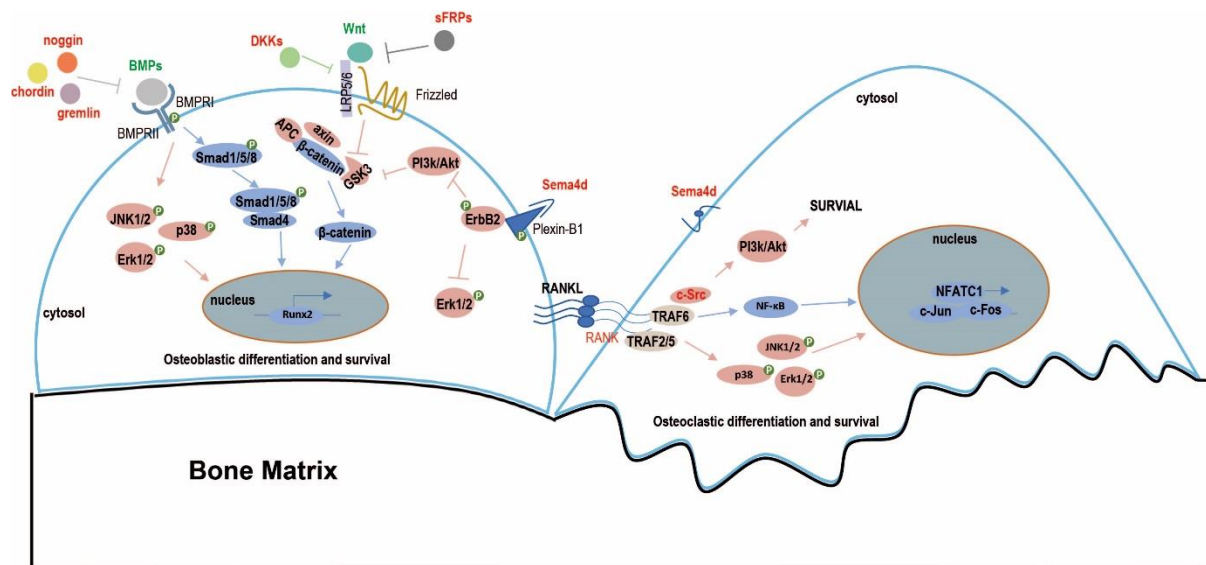


Fig. 1. Selective signaling pathways involved in bone homeostasis. During osteogenesis, BMPs and Wnts induce MSCs to differentiate into osteoblasts, which can be inhibited by extracellular antagonists such as noggin, chordin, gremlin, DKKs, and sFRPs. In the aspect of osteoclastogenesis, hematopoietic cells differentiate into osteoclasts under RANKL stimulation. The osteogenesis and osteoclastogenesis are mutually modulated. Osteoblastic lineages secreted RANKL increases osteoclastic generation and activity. The Sema4d expressed from osteoclasts inhibits osteoblasts activity. On the other hand, osteoclasts digest bone matrix resulting in osteogenic factors release.

2. RNAi-based therapeutics for osteogenesis

Compared with conventional pharmacological inhibitors, siRNA offer several advantages such as high specificity and facile synthesis (Ozcan, Ozpolat et al. 2015). siRNA can be designed to target a specific gene which is particularly useful to alter signaling pathways [(Miller, Xia et al. 2003)], even proteins inaccessible to conventional drugs [(Filleur, Courtin et al. 2003); (Dorn, Patel et al. 2004)].

In the following section, we focus on the therapeutic approach using siRNA mediated gene silencing to accelerate osteogenesis

2.1 siRNA mechanism of action

The RNA interference (RNAi) is a conserved mechanism regulating endogenous gene expression and defending against intracellular pathogens such as viruses [(Agrawal, Dasaradhi et al. 2003)]. The term of RNAi was coined after the discovery that the injected dsRNA lead to specific silencing of genes highly homologous in sequence to the injected dsRNA in *Caenorhabditis elegans* [(Fire 1999)]. Soon after, Elbashir and colleagues revealed RNAi in mammalian cells [(Elbashir, Harborth et al. 2001)].

miRNAs are conserved small non-coding dsRNAs transcribed by genomes that exert RNAi functions through silencing the target mRNAs [(Hannon 2002)]. siRNAs are synthetic dsRNAs designed to silence a specific gene, which has 21-23 nucleotides with 3' 2-nucleotides overhangs and 5' phosphate groups [(Elbashir, Lendeckel et al. 2001)]. The siRNAs can be exogenously introduced into cells in final short form or long dsRNA form which cleaved by Dicer, an RNase III-like enzyme, in cytoplasm to generate functional siRNAs. Interacting with AGO2-RISC enzyme complex, the sense strand of the siRNA is cleaved, and the anti-sense remains associated with the complex. The anti-sense guides the RISC complex binds to the targeting mRNA with full complementarity which then cleaved by AGO2. Fig. 2 exhibits the gene silencing process mediated by siRNA.

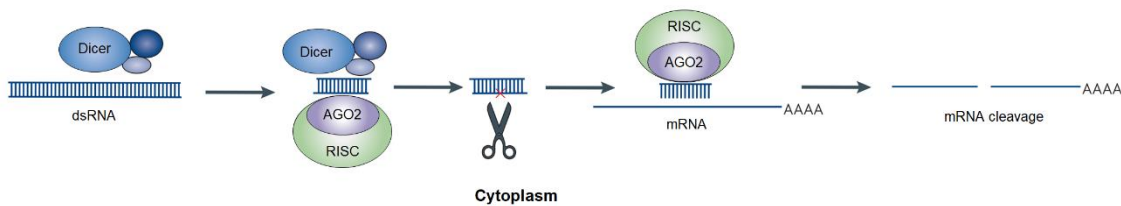


Fig. 2. Schematic mechanism of siRNA mediated RNA interference in mammalian cells. In cytoplasm, the Dicer enzyme cleaves long double-stranded RNA (dsRNA) to form active siRNA. The siRNA interacts with Argonaute 2 (AGO2) and the RNAi-induced silencing complex (RISC) leading to cleavage of the sense strand by AGO2. The target mRNA with complementary sequence to the antisense strand is recognized and cleaved. Reproduced from (de Fougères, Vornlocher et al. 2007) with permission.

2.2.1 Silencing BMPs antagonists:

a) noggin siRNA

Among the BMP antagonists, noggin is identified to be the most closely involved in the osteoinductive process of BMPs [(Smith and Harland 1992); (Abe, Yamamoto et al. 2000)]. Several studies demonstrated that increase noggin expression significantly impairs bone formation both *in vitro* and *in vivo* [(Devlin, Du et al. 2003); (Xiao, Gopalakrishnan et al. 2002); (Wu, Li et al. 2003)]. These studies reinforce the crucial role of noggin as a negative regulator of bone formation. Wan and colleagues were the pioneers of this strategy. Retroviral particles encoding noggin siRNA were used for *in vitro* and *in vivo* experiments [(Wan, Pomerantz et al. 2007)]. *In vitro* introduction of noggin siRNAs into MC3T3-E1 resulted in approximately 90% noggin transcripts reduction. Noggin elimination increased the Smad1/5 phosphorylation inducing BMP signaling, which results to the increase of calcium deposition. *In vivo* strategy was based on primary calvarial osteoblasts transduced with noggin siRNA soaked into apatite-coated polylactic-co-glycolic acid

scaffolds before implantation into a mice calvarial defect. After 2 weeks, the group of mice implanted with noggin down expressing cells demonstrated around 6-fold more bone content than the vehicle control group. Reducing noggin levels enhances both intracellular and extracellular activities of BMPs, which is of benefit compared to BMPs administration requiring supraphysiological BMP doses, always associates with some side effects (Shimer, Oner et al. 2009). Indeed, noggin expression was upregulated when recombinant BMP-2 was used to stimulate murine C2C12 myoblasts and mesenchymal stem cells (MSC), which in turn inhibits the osteogenic function of BMP-2. (Takayama, Suzuki et al. 2009); (Chen, Uludag et al. 2012). Studies from Takaoka's group demonstrated that noggin siRNA delivery abrogated rhBMP-2 induced noggin expression, resulting in accelerated osteoblastic differentiation of C2C12 cells *in vitro* and ectopic bone formation *in vivo* compared to rhBMP2 alone [(Takayama, Suzuki et al. 2009); (Manaka, Suzuki et al. 2011)]. Manaka and colleagues used a biodegradable hydrogel made from poly-D, L-lactic acid-p-dioxanonepolyethylene glycol block copolymer (PLA-DX-PEG) for noggin siRNA *in vivo* delivery. Implant of noggin siRNA and rhBMP2 containing PLA-DX-PEG hydrogel into a mice dorsal muscle pouch successfully inhibited rhBMP2 induced noggin expression compared to hydrogel blend with rhBMP2 and control siRNA. The suppression effect lasts for up to 7 days with the peak occurring at day 2 (Manaka, Suzuki et al. 2011).

The function of noggin silencing to benefit bone regeneration was improved when high efficient vector to deliver noggin siRNA was used (Choi, Cui et al. 2015); (Inada, Tamura et al. 2018). As an example, Cui and colleagues developed a sterosome vector composed of stearylamine (SA) and cholesterol (Chol) for delivering noggin siRNA to enhance the osteogenic differentiation of AMSCs (Cui, Fan et al. 2015). The freshly prepared SA/Chol/siRNA nanoparticles (NPs) exhibited high transfection efficiency (93%) comparable to commercial vector lipofectamine 2000® (96%). The value decreased to approximately 60% after incubating at 37°C for 2 weeks, while it was highly reduced to $0.2 \pm 0.1\%$ for lipofectamine 2000® demonstrating that SA/Chol/siRNA NPs has excellent colloidal stability. Cells transfected with SA/Chol/noggin siRNA showed more than 45% noggin expression reduction significantly higher than that obtained using lipofectamine 2000® (~25%). In a mouse calvarial defect model, methacrylated glycol chitosan (MeGC) hydrogel encapsulating AMSCs transfected with SA/Chol/noggin siRNA particles was implanted into the defect site. Defects treated with SA/Chol/noggin siRNA were filled with ~20% regenerated bone tissue which are ~15% and ~5% for using lipofectamine 2000/siRNA groups and control siRNA groups, respectively. This example point out the discrepancy that could be obtained between the *in vitro* and *in vivo* efficiency.

b) chordin and gremlin siRNAs

Silencing chordin and gremlin could lead as well to enhanced osteogenesis *in vitro* and *in vivo*. The study from Kwong et al. firstly demonstrated that delivery of siRNA against chordin accelerates BMSCs osteogenic differentiation (Kwong, Richardson et al. 2008). Comparing the expression of noggin, gremlin, chordin in bone marrow stem cells (BMSCs) isolated from patients with normal fracture healing and those with bone nonunion (BMSCs/N), Wang et al. found that chordin expression was the most upregulated (Wang, Xiao et al. 2018). Silencing chordin in BMSCs/N yielded more expression of osteogenic markers than gremlin and noggin silencing. Based on these observations, the authors defined chordin as the ideal target for inducing BMSc/N osteogenic differentiation. Next, they tested different vehicles, i.e. polyspermine imidazole-4,5-imine (PSI), Polyethyleneimine of various size (800 and 25kDa) and spermine, to optimize chordin siRNA delivery. Results of *in vitro* osteogenic differentiation and *in vivo* bone regeneration demonstrated that PSI was the most potent vector followed by PEI 25KDa.

Interestingly, transfecting ST-2 stromal cells with gremlin siRNA using siLentFect[®] significantly enhanced the stimulatory effect of BMP-2 on alkaline phosphatase expression at all BMP-2 doses (0-10nM) tested (Gazzerro, Smerdel-Ramoya et al. 2007). Such enhancement relied on increased Smad1/5/8 phosphorylation. Moreover, gremlin silencing enhanced as well Wnt3a signaling. A recent study showed gremlin knockdown by lipofectamine 2000[®]/gremlin-siRNA enhanced human MSCs osteogenic differentiation [(Hu, Sun et al. 2017)]. To date, no *in vivo* published studies using gremlin siRNA for bone formation.

2.2.2 Silencing osteoclast stimulants and osteoblasts inhibitors

During osteoporosis, overactivation of osteoclasts results in the decreased bone mass and high-risk bone fracture that dramatically influence the quality of life. Osteoblast-osteoclast communication is mediated by several pairs of ligands and receptors which are potential therapeutical targets to modulate bone regeneration. RANK/RANKL is known to be a critical signaling initiating osteoclast formation and activity. Silencing RANK gene with RANK siRNA successfully inhibited osteoclast differentiation of RAW264.7, a murine-macrophage osteoclast precursor cell line, *in vitro* (Wang and Grainger 2010); (Wang, Tran et al. 2012); (Ma, Yang et al. 2014); (Sezlev Bilecen, Uludag et al. 2019). Transfecting RAW cells with DharmaFECT4/ RANK-siRNA complexes, Wang and colleagues as pioneers, suppressed osteoclastic RAW cells as well as primary osteoclast precursors differentiation from mouse bone marrow cells. Two transfection strategies were tested for RANK silencing which are one single transfection, and 3 successive transfusions performed every other day. Both showed significant reduction of RANK expression and osteoclast formation, especially upon multiple transfusions. In the study, osteoclast-mediated bone

resorption were measured by seeding cells onto bovine bone slices. Results showed that the resorption pit numbers on bone slices were markedly curtailed in the RANK-siRNA treated groups.

Proto-oncogene tyrosine-protein kinase Src (c-Src) is a factor downstream RANK signaling controls bone metabolism by maintenance of osteoclast function [(Boyce, Yoneda et al. 1992); (Lowe, Yoneda et al. 1993)]. Knockdown c-Src in mice showed increased bone formation leading to osteopetrosis [(Frattini, Orchard et al. 2000)]. Zhang et al. injected jetPEI®/Src-siRNA complexes into the proximal femur in a corticosteroid-induced osteoporosis rabbit model [(Zheng, Wang et al. 2016)]. Src siRNA administration reduced Src mRNA level to ~50% in bone marrow cells of treated rabbits. Micro-computed tomography (μCT) analysis revealed that Src siRNA prevented bone loss in metaphyseal trabecular and maintained the bone mineral density similar to normal rabbit, whereas non-Src targeting siRNA groups and vehicle control group showed remarkably lower bone mineral densities.

Semaphorin 4d (Sema4d) is a recently uncovered molecule involved in osteoclast-osteoblast communication (Negishi-Koga, Shinohara et al. 2011). Sema4d secreted from osteoclasts reduced the osteoblastic differentiation of cocultured BMSCs, which was reversed by knockdown Sema4d in osteoclasts by Sema4d siRNA [(Zhang, Wei et al. 2015)]. The negative regulation property to osteoblast activity makes Sema4d a targeting candidate to increase osteogenesis. Intravenously delivery of Sema4d siRNA complexed with a bone targeting polymer, N-(2-hydroxypropyl)methacrylamide (HPMA) copolymers with D-aspartic acid octapeptide (D-Asp8), to murine osteopenia model resulted in improved bone mineral density, bone volume, and trabecular thickness (Zhang, Wei et al. 2015). The Sema4d siRNA particles also worked to reverse periodontal disease induced alveolar bone loss (Zhang, Wei et al. 2014).

Table 1 . Summary of representative studies based on siRNA for *in vivo* osteogenesis.

antagonist	Target	Vector	In vivo delivery	in vivo model	Ref.
Local delivery					
Chordin	BMP2	PSI; PEI800; PEI25KDa; spermine	Matrigel/transfected hBMSCs	Mouse tibial monocortical defect	(Wang, Xiao et al. 2018)
		PEI-Et; PEI800; PEI25KDa	Matrigel/transfected hBMSCs	Mouse tibial monocortical defect	(Wang, Yuan et al. 2017)
retrovirus		Apatite coated PLGA scaffold /calvarial osteoblasts	Mouse Calvaria defect	(Wan, Pomerantz et al. 2007)	
Lipofectamine 2000 ^{○,R}		dorsal muscle injection followed by electroporation	Mouse ectopic bone formation-dorsal muscle	(Takayama, Suzuki et al. 2009)	
Noggin					

		Naked siRNA	Paraspinal muscle injection followed by electroporation	Rabbit posterolateral spinal fusion	(de Fougères, Vornlocher et al. 2007)
		Naked siRNA	PLA-DX-PEG hydrogel/ siRNA	Mouse ectopic bone formation-dorsal muscle	(Manaka, Suzuki et al. 2011)
		Sterosomes	methacrylated glycol chitosan hydrogel/ siRNA	Mouse Calvaria defect	(Cui, Fan et al. 2015)
		PEI	PEG hydrogel/ siRNA	rat calvarial defect	(Nguyen, Jeon et al. 2018)
		mPEG-bPEI-PAsp(DIP-BzA)	Fibrin hydrogel/ transfected MC3T3-E1 cells	mouse calvarial defect	(Huang, Lin et al. 2018)
GNAS1	Runx2	ND98	PLGA fibrous membrane/ siRNA/ hADSCs	Mouse calvaria defect	(Shin, Cho et al. 2016)
		Ambion siPort ^{○,R}	Silk fibroin-chitosan scaffold/ siRNA	Sheep ectopic bone formation-latissimus dorsi muscle	(Rios, Skoracki et al. 2012)
Sema4d	Osteoclast-osteoblast communication	Asp8-Stearyl-R8	PLLA scaffold/ siRNA	3 mm femoral cylindrical defects in ovariectomized rat	(Zhang, Wei et al. 2016)
Src	Osteoclast-osteoblast communication	jetPEI ^{○,R}	intramedullary injected into proximal femur	corticosteroid-associated osteoporotic rabbit	(Zheng, Wang et al. 2016)
Systemic delivery					
Sema4d	Osteoclast-osteoblast communication	Asp8-Stearyl-R8	IV	ovariectomized osteoporotic mouse	(Zhang, Wei et al. 2015)

PEI: polyethylenimine; PSI: polyspermine imidazole-4,5-imine; PEI-Et: ethylene bis linked low-molecular-weight polyethylenimine; GNAS1: guanine nucleotide-binding protein alpha-stimulating activity polypeptide 1; mPEG-bPEI-PAsp(DIP-BzA): monomethoxy-poly(ethylene glycol)-b-branched polyethyleneimine-b-poly(N-(N',N'-diisopropylaminoethyl)-co-benzylamino)aspartamide; PLA-DX-PEG: poly-D,L-lactic acid-p-dioxanonepolyethylene glycol block co-polymer; ND98: five-tailed isomer of triethylenetetramine-laurylaminopropionate with a free internal amine, Cholesterol, and mPEG2000-C14 Glyceride; PLLA: poly-L-lactic acid; Asp8-Stearyl-R8: D-aspartic acid Octapeptide grafted Stearyl octaarginine; IV: intravenous; Sema4d: semaphorin 4d.

2.3 Challenges and perspectives

siRNA-based therapeutics hold great promises as they can virtually silence any genes, which may be inaccessible with conventional small molecules or protein drugs. In August 2018, FDA approved the first

ever siRNA against transthyretin mRNA (Patisiran) responsible of hATTR, a life-threatening neurodegenerative rare inherited disease opens a real new type of pharmaceuticals ((Adams, Suhr et al. 2017)). Today, many advances in design of siRNA have permitted this this landmark achievement. The first challenge is to design a rational siRNA sequence with high silencing efficiency, without off-target effects and immunogenicity. The off-target occurs when the siRNA has partial complementarity in the seed region with unintended genes. Moreover, siRNAs complementary to different regions of the target mRNA could result to diverse silencing efficiency. Today, there are siRNA design algorithms that can help to predict the silencing efficacy and reduced off-target effect ([Chaudhary, Srivastava et al. 2011](#)) ([Naito and Ui-Tei 2013](#)). siRNA is also known to induce innate immune system in mammals (([Kariko' et al. 2004a](#), [Sioud and Sørensen 2003](#)). It results in induction of interferon (IFN) and pro-inflammatory cytokines production. Duplexes of siRNA can be sensed by PKR and TLR3/7/8, which can be reduced by chemical modifications, e.g. ribose 2'-OH group modification, locked and unlocked nucleic acids modification, and backbone phosphorothioate modification ([Lam, Chow et al. 2015](#)).

The second challenge is the delivery of siRNA to the target sites in an efficient and safe manner. siRNAs share the same types of non viral delivery systems with other nucleic acids, which are polymer-based vectors, lipid-based vectors, and inorganic vectors. Despite the progress made in improved transfection efficiency and reduced toxicity, delivering therapeutic siRNA for osteogenesis meets unique barrier. For optimal therapeutical effects, bone targeting vectors are needed. Some ligand moieties showed bone targeting ability such as bisphosphonates, oligopeptides (Asp)₈ and (AspSerSer)₆, and aptamers which have high affinity to calcium in hydroxyapatite, crystalline hydroxyapatite, amorphous hydroxyapatite, and osteoblast, respectively ([Liu 2016](#)). Bone-specific delivery systems were obtained by conjugating those moieties to delivery systems and their use resulted to some promising results ([Zhang, Wei et al. 2016](#)) ([Zhang, Wei et al. 2015](#)). Covalent Tetracyclines (TCs), conjugation with poly(lactic-co-glycolic acid) (PLGA) copolymer (TC-PLG1) could be another delivery system alternative ([Wang, Liu et al. 2015](#)). TC is an antibiotic agent that has been found to be taken up by bone via its interaction with hydroxyapatite Ca₁₀(PO₄)₆(OH) (([Perrin 1965](#))). PLGA is an FDA approved biodegradable polymer that is widely used to delivery therapeutic molecules including nucleic acids ([Wang, Tran et al. 2012](#)). In ovariectomized osteoporosis rat model, TC-PLGA nanoparticles have a tendency to accumulate in bone compared to PLGA alone ([Wang, Liu et al. 2015](#)). When loaded with simvastation, TC-PLGA particles were more potent to enhance bone mineral density over PLGA. It could be worthwhile to exploit similar strategy for siRNA.

3 mRNA-based therapeutic for osteogenesis

3.1. Eukaryotic mRNA and *in vitro* transcription

mRNAs are the intermediate between the genetic information stored in DNA and proteins. DNA is first transcribed into mRNA in the nucleus before mRNA exports and translation into proteins by the ribosomes in the cytoplasm. All mature eukaryotic mRNA consists of five moieties, which are the 5' methylated guanosine cap (m^7Gp3N), the 5' and 3' untranslated region (5'-UTR and 3'-UTR) flank the open reading frame (ORF), and the 50-250 adenosine residues (poly(A) tail) [(Jackson 1993); (Banerjee 1980)] (Fig. 3). ORF stores protein information in the codon sequence while the rest moieties responsible for regulation of mRNA translocation, translation, and stability. Understanding the mechanisms behind mRNA translation and stability regulation has been useful to guide the design of *in vitro* transcribed (IVT) mRNA and increase the efficacy of mRNA therapy.

The 5' m^7G cap is an evolutionarily conserved modification of eukaryotic mRNA with an N^7 methylated guanosine covalent to the first nucleotide of the pre-mRNA *via* a reversed 5' to 5' triphosphate linkage. Additional to N^7 methylation in 5' m^7G cap (m^7GpppN , Cap 0), in higher eukaryotes, methylation also occurs at the 2'-O position of the +1 ribonucleotide ($m^7GpppNm$, Cap 1) and +2 ribonucleotide ($m^7GpppNmNm$, Cap 2), respectively [(Wei, Gershowitz et al. 1975);(Langberg and Moss 1981)]. The cap structure and properties were revealed in the 1970s, due to the technical development of purification mRNA based on the poly(A) structure (Edmonds, Vaughan et al. 1971); (Mendecki, Lee et al. 1972). The majority of cellular mRNA translation is initiated in a cap-dependent manner. The cap acts as an anchor for recruiting eukaryotic translation initiation factor 4F complex (eIF4F complex), including eIF4E, eIF4G, and eIF4A, which is responsible for the recruitment of 43S complex of the small ribosomal subunit and associated translation factors to form the 48S pre-initiation complex (McCormick and Khapersky 2017). mRNA lacks m^7G cap exhibited at least ten folds protein expression reduction (Horikami, De Ferra et al. 1984); (Lo, Huang et al. 1998). Cap analogs, such as GpppG, m^7GpppG and $m^7GpppGm$, are incorporated into RNAs during IVT (Konarska, Padgett et al. 1984); (Contreras, Cheroutre et al. 1982). However, one-third of the IVT mRNA is improperly capped thus poorly recognized by the translation initiation machinery [(Matsuo, Li et al. 1997); (Cai, Jankowska-Anyszka et al. 1999)]. To address this problem, the Jemielity group developed the anti-reverse cap analog (ARCA) in which the hydroxyl group in C2 or C3 position of the m^7G is removed or substituted with methoxy group [(Stepinski, Waddell et al. 2001); (Jemielity, Fowler et al. 2003)]. The ARCA-capped mRNA resulting in increased as well as prolonged protein expression [(Mockey, Goncalves et al. 2006); (Zohra, Chowdhury et al. 2007)]. A further improvement in synthetic

cap analogs has been achieved using sulfur to substitute a nonbridging oxygen in either the α , β , or γ -position of the triphosphate. This substitution leads to an inhibition of pyrophosphatase Dcp1p/Dcp2p mediated mRNA decapping degradation ([Grudzien-Nogalska, Jemielity et al. 2007](#)). Another option is to replace the triphosphate with tetraphosphate to increase the affinity to translation initiation factor eIF4E to the cap analog ([Strenkowska, Kowalska et al. 2010](#)).

5' and 3' UTRs play crucial roles in mRNA regulation of translocation ([Jansen 2001](#)), translation ([van der Velden and Thomas 1999](#)) and stabilization ([Bashirullah, Cooperstock et al. 2001](#)). 5'-UTR regulates mRNA translation efficiency through its structure features, i.e., length, secondary structure, nucleotide sequence ([Mignone, Gissi et al. 2002](#)). The best context for translation initiation is when the starting methionine codon (AUG) is in a Kozak sequence context ($GCC^A/_6CCAUGG$) with a purine at the -3 position and a guanine at the +4 position ([Kozak 1987](#)), ([Kozak 2005](#)). Yang et al. found that mRNAs with longer 3'-UTR (>1Kb) decayed at a significantly faster rate than shorter 3'-UTR ([Yang, van Nimwegen et al. 2003](#)). Additionally, the exosome complex destructs transcripts bearing AU-rich motifs ([Chen, Gherzi et al. 2001](#)); ([Mukherjee, Gao et al. 2002](#)), especially when the AU-rich motif is located in the 3'-UTR ([Yang, van Nimwegen et al. 2003](#)).

Poly (A) tail is also essential to improve mRNA intracellular stability and for successful translation initiation. Initiation of translation requires the formation of a pre-initiation complex (PIC) which is Poly(A) tail binds to polyadenylate-binding protein 1 (PABP) then interacts with eIF4G leading to mRNA circulation ([Gallie 1998](#)). The length of the poly(A) tail is essential for mRNA stability. Eukaryotic mRNA undergoes progressive deadenylation leading to decapping followed by 5'-3' exonucleolytic degradation ([Coller and Parker 2004](#)). Transcripts with a short poly(A) tail are shown more vulnerable to decapping ([Couttet, Fromont-Racine et al. 1997](#)). In parallel, translation efficient of administered IVT mRNA is positively correlated with poly(A) tail length ([Mockey, Goncalves et al. 2006](#)); ([Peng, Murray et al. 2008](#)). The poly(A) tail could be either put on the template or added post IVT by enzymatic polyadenylation; the former strategy resulting in less polydisperse mRNA with higher translational activity ([Grier, Burleigh et al. 2016](#)).

In summary, the IVT mRNA should at least contains a cap structure, a poly(A) tail longer than 12 A residues which is the minimum poly(A) length for PABP binding ([Sachs, Davis et al. 1987](#)). For optimal expression and stability, the 5' and 3' UTR with designed sequence can be added.

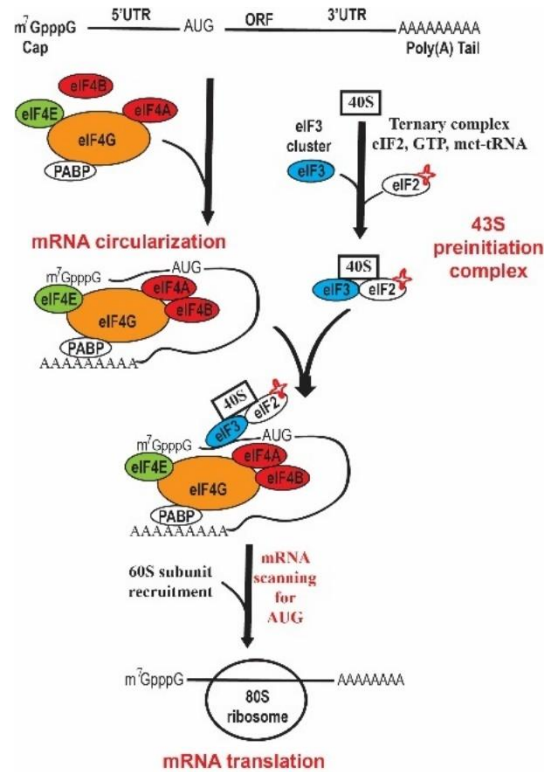


Fig. 3: Eukaryotic mRNA structure and translation initiation. Mature eukaryotic mRNA consists the 5' methylated guanosine cap (m^7Gp3N), the 5' and 3' untranslated region (5'-UTR and 3'-UTR) flank the open reading frame (ORF), and the 50-250 adenosine residues (poly(A) tail). During translation, eukaryotic translation initiation factor 4E (eIF4E) binds to the m^7Gp3N cap, and polyadenylate-binding protein 1 (PABP1) binds to the 3' poly(A) tail, followed by recruitment of eIF4G and eIF4A, leading to mRNA circularization. The translation initiation factor 4F complex (eIF4E, eIF4G, and eIF4A) recruits 43S preinitiation complex and associated translation factors to the 5'-UTR. Next, $tRNA^{met}$ recognizes the initiation codon (AUG), and large ribosome subunit (80S) locks the ORF and starts polypeptide synthesis.

3.2 mRNA therapy and immunogenicity

Introducing exogenous mRNA into cells to express protein of interest started three decades ago. In 1989, Malone and colleagues transfected tissue cultured NIH3T3 cells with IVT *P. pyralis* luciferase mRNA using a synthetic cationic lipid (Malone, Felgner et al. 1989). The following year, Wolff and colleagues first demonstrated the feasibility of IVT mRNA mediated protein expression *in vivo* by direct injection of naked IVT mRNAs into mouse skeletal muscle [(Wolff, Malone et al. 1990)]. In 1992, arginine vasopressin IVT mRNA was used to treat genetic mutation of vasopressin-induced diabetes insipidus in rat (Jirikowski,

Sanna et al. 1992). The diabetes insipidus was temporarily reversed for up to 5 days. Since then, extensive studies were focused on mRNA therapy (Tavernier, Andries et al. 2011)(Schlake, Thess et al. 2018). Although mRNA design, synthesis, and delivery methods gained remarkable progress, the use of mRNA has been so far limited to vaccination because of its instability and immunogenicity [(Pascolo 2008); (Schlake, Thess et al. 2018)]. After internalization, mRNA is mainly accumulated into endosomes, where the IVT mRNAs are recognized by Toll-like receptors (TLRs), specifically TLR3, 7, and 8, located in the endosome membrane [(Kariko, Buckstein et al. 2005); (Alexopoulou, Holt et al. 2001); (Heil, Hemmi et al. 2004)]. Binding of RNA to TLR results in the induction of an antiviral response resulting in RNA degradation and translational shutoff. TLR3 recognizes double-stranded RNA, and TLR7/8 recognize single-stranded RNA. The TLRs stimulation induces type I interferon (INF α/β) expression and associated anti-viral responses resulting in IVT mRNA translation suppression and degradation, and even host cell apoptosis [(Kariko, Ni et al. 2004); (Dan, Zheng et al. 2012)]. The comprehensive immune responses induced by IVT mRNA is shown in Figure 4.

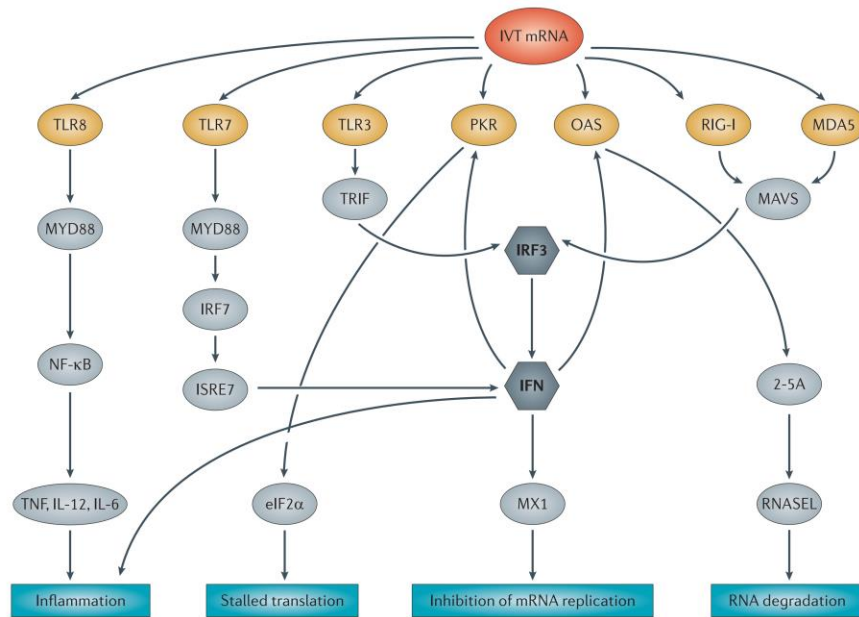


Fig. 4. Immune responses to synthetic mRNA. In vitro transcribed (IVT) mRNA is recognized by various endosomal innate immune receptors (Toll-like receptor 3 (TLR3), TLR7 and TLR8) and cytoplasmic innate immune receptors (protein kinase RNA-activated (PKR), retinoic acid-inducible gene I protein (RIG-I), melanoma differentiation associated protein 5 (MDA5) and 2'–5'-oligoadenylate synthase (OAS)). Signaling through these different pathways results in inflammation associated with type 1 interferon (IFN), tumor necrosis factor (TNF), interleukin-6 (IL-6), IL-12 and the activation of cascades of

transcriptional programmers. Overall, these create a pro-inflammatory microenvironment poised for inducing specific immune responses. Moreover, downstream effects such as slow-down of translation by eukaryotic translation initiation factor 2 α (eIF2 α) phosphorylation, enhanced RNA degradation by ribonuclease L (RNASEL) overexpression and inhibition of replication of self-amplifying mRNA are of relevance for the pharmacokinetics and pharmacodynamics of the IVT mRNA. IRF, interferon regulatory factor; ISRE7, interferon-stimulated response element; MAVS, mitochondrial antiviral signaling protein; MDA5, melanoma differentiation-associated protein 5; MYD88, myeloid differentiation primary response protein 88; MX1, myxovirus (influenza) resistance 1; NF- κ B, nuclear factor- κ B; TRIF, Toll-IL-1 receptor domain-containing adapter protein inducing IFN β . Adapted from (Sahin, Kariko et al. 2014) with permission.

In the last 10 years, the discovery that incorporation of naturally modified nucleotides, such as pseudouridine, 5-methylcytidine, N6-methyladenosine, 5-methyluridine, or 2-thiouridine, suppressed IVT mRNA induced TLR3/7/8 activation in human DCs revitalized mRNA-based therapies [(Kariko, Buckstein et al. 2005); (Freund, Eigenbrod et al. 2019)]. Further, Kariko and colleagues compared the influence of each modified nucleotides in mRNA translation [(Kariko, Muramatsu et al. 2008)]. Interestingly, although pseudouridine is primarily found in tRNA, rRNA, and small nuclear RNA (Anderson, Muramatsu et al. 2010), incorporation of pseudouridine into mRNA showed the highest translation enhancement *in vitro*, because pseudouridine incorporation changed the mRNA structure motifs which can active RNA-dependent protein kinase (PKR) leading to inhibition of translation initiation by eIF2 α phosphorylation [(Zheng and Bevilacqua 2004)]. In addition to the nucleotides mentioned above, other modified nucleotides also exhibited the ability to increase mRNA translation and stability (Li, Luo et al. 2016). Svitkin and colleagues showed N1-methyl-pseudouridine modified luciferase mRNA yield 7.4-fold luciferase expression compared to non-modified mRNA, which was higher than the 5-methylcytidine modification and their combination. In addition, they described the enhanced translation was partially contributed by suppressed eIF2 α phosphorylation and increased ribosome pausing and density on the mRNA [(Svitkin, Cheng et al. 2017)].

mRNA obtained by IVT is not uniform in length and structure. They can contain contaminants such as short RNAs generated from abortive transcription and double-stranded RNAs generated from RNA-primed transcription from RNA templates, self-complementary 3' extension, transcription runoffs and, RNA-dependent RNA polymerase activity [(Milligan, Groebe et al. 1987); (Triana-Alonso, Dabrowski et al. 1995); (Arnaud-Barbe, Cheynet-Sauvion et al. 1998); (Nacheva and Berzal-Herranz 2003); (Mu, Greenwald et al.

2018). These contaminants are potent inducers of immune responses and their removal requires mRNA purification after IVT [(Kariko, Muramatsu et al. 2008); (Kariko, Muramatsu et al. 2011)]. The purification is mostly done using chromatography to separate appropriately-sized mRNA from free nucleoside triphosphates, short RNA and DNA template [(Kim, McKenna et al. 2007); (McKenna, Kim et al. 2007)]. The elimination of double-stranded RNA requires high-performance liquid chromatography (HPLC) purification [(Kariko, Muramatsu et al. 2011); (Weissman, Pardi et al. 2013)]. These procedures are now used by most of mRNA-producing companies.

3.3 mRNA-based therapeutic for osteogenesis

mRNA as a potential therapeutic extends from vaccination to protein replacement therapy once modified mRNA was shown to facilitate *in vivo* exogenous mRNA-mediated protein expression with no or low immunogenicity [(Kariko, Muramatsu et al. 2008); (Kormann, Hasenpusch et al. 2011)]. Zangi et al. injected modified RNA encoding human VEGF-A into mouse heart with myocardial infarction [(Zangi, Lui et al. 2013)]. The VEGF-A expressed from modified mRNA significantly decreased the infarct size and enhanced the survival rate of recipients.

3.3.1 BMPs replacement therapy

For bone regeneration, recombinant BMPs have been used in therapy for over 40 years (Urist and Strates 2009). As dominant osteoinductive factors, BMPs have been delivered into the site that needs bone regeneration in the form of recombinant protein [(McKay, Peckham et al. 2007)] or via plasmid encoding BMP gene used as naked or vectorized form (Fang, Zhu et al. 1996);(Khorsand, Nicholson et al. 2017). The outcomes were promising, and the drawbacks were also apparent, as mentioned previously. Elangovan et al. employed modified mRNA encoding BMP-2 to enhance bone regeneration *in vitro* and *in vivo* (Elangovan, Khorsand et al. 2015). Using IFN α as a marker, combined modification of pseudouridine and 5-methylcytidine almost entirely circumvented unmodified mRNA induced immune response. They compared the osteoblastic differentiation induction between the modified BMP2 mRNA and its pDNA counterpart. Bone Marrow-derived Stem Cells (BMSCs) transfected with polyethylenimine (PEI)-complexed modified mRNA (branched PEI, mol. wt. 25 kDa) yielded three times more BMP2 in the cell culture medium than cells transfected with pDNA. The osteogenic differentiation markers expression, such as alkaline phosphatase (ALP), osteocalcin (OCN), were also higher in the modified mRNA group. As a result, implanting a collagen scaffold containing modified mRNA polyplexes into rat calvarial defects showed 3.94-fold higher BV/TV (mineralized bone volume as a fraction of the total tissue volume of

interest) in defect site compared to empty scaffold control. This study, for the first time, verified the safety and efficacy of using modified mRNA coding an osteogenic factor for bone regeneration. It also demonstrated the potential of modified mRNA-loaded scaffold as a novel bone graft substitute. Similarly, BMP-2 mRNA bearing methylcytidine and 2-thiouridine modification exhibited greater bone formation capacity [(Balmayor, Geiger et al. 2016); (Badiayan, Berezhansky et al. 2016); (Balmayor, Geiger et al. 2017)]. Balmayor et al. prepared modified mRNA coding human BMP-2 by replacing 25% cytidine and 25% uridine with methylcytidine and 2-thiouridine. Adipo-derived mesenchymal stem cells (AMSCs) showed osteogenic differentiation once transfected with this modified BMP-2 mRNA through lipofection or magnetofection (Badiayan, Berezhansky et al. 2016). Interestingly, AMSCs osteogenic differentiation was dose-dependent. For *in vivo* osteogenesis, liposomal formulation of modified BMP-2 mRNA mixed with a fibrin matrix was implanted into rat femur hole model. Two weeks post-operation, rats treated with modified BMP-2 mRNA obtained ~2-fold more callus tissue than fibrin matrix alone.

Most of studies on bone regeneration have been based on the use of BMP2 since it is considered as one of the most osteogenic factors. A recent study highlighted that BMP-9 could be also used as an alternative. The efficiency of modified mRNA coding BMP-2 or BMP-9 to induce *in vitro* osteogenic induction and *in vivo* bone regeneration have been compared in a recent report (Khorsand, Elangovan et al. 2017). BMSCs transfected with PEI-complexed modified BMP-9 had a significant increase in ALP gene expression and calcium deposition compared to modified BMP-2 mRNA, but not in Runx2 and OCN genes expression. Implanting collagen matrices containing modified BMP-9 mRNA or BMP-2 into rat calvarial defect resulted in a similar ratio of bone volume to tissue volume. However, modified BMP-9 groups had 2-fold more density in connective tissue than BMP-2 groups, which is consistent with the *in vitro* observation.

Table 2: Summary of published studies using mRNA for *in vivo* bone regeneration.

Gene	Nucleotides modification in IVT mRNA	vector	Scaffold/cells	In vivo model	Ref.
BMP-2	$\psi_{(1)}^*$ and m5C ₍₁₎	Branched PEI (25 kDa)	Collagen sponge containing 25 μ g mRNA polyplexes	Rat calvarial defect-5 mm diameter	(Elangovan, Khorsand et al. 2015)
BMP-2	s2U _(0.25) and m5C _(0.25)	cationic lipid C12-EPE with helper lipids DOPE, cholesterol, and DMPE-PEG 2k	Fibrin gel loaded with 2.5 μ g mRNA lipoplexes	Rat femur diaphysis transcortical bone defect-2mm diameter	(Balmayor, Geiger et al. 2016)

BMP-2	s2U _(0.25) and m5C _(0.25)	cationic lipid C12-EPE with helper DPPC and cholesterol, and DMG-PEG2K	Collagen sponge containing 2.5 µg mRNA lipoplexes	Rat femur diaphysis transcortical bone defect-2mm diameter	(Badieyan, Berezhanskyy et al. 2016)
BMP-2/9	ψ ₍₁₎ and m5C ₍₁₎	Branched PEI (mol. wt. 25 kDa)	Freeze dried collagen scaffold containing 50 µg mRNA polyplexes	Rat calvarial defect-5 mm diameter	(Khorsand, Elangovan et al. 2017)
BMP-9	ψ ₍₁₎ and m5C ₍₁₎	Branched PEI (mol. wt. 25 kDa)	Freeze dried collagen membrane containing 10 µg mRNA polyplexes	Rat calvarial defect-5 mm diameter	(Khorsand, Elangovan et al. 2018)
BMP-2	5IU _(0.35) 5IC _(0.075)	cationic lipid C12-(2-3-2) with helper lipids DPPC, cholesterol, and DMG-PEG 2k	Vacuum dried collagen sponge containing 2.5 µg or 5 µg mRNA lipoplexes	Rat femoral segmental bone defect-5mm diameter	(Zhang, De La Vega et al. 2018)

*: the subscripted value represents the proportion of modified nucleotides in the IVT mRNA; Ψ: pseudouridine; m5C: 5-methylcytosine; 5IU: 5-iodo-uridine; 5IC: 5-iodo-cytidine; s2U: 2-thiouridine; C12-EPE: DOPC: 1,2-Dioleoyl-sn-Glycero-3-Phosphocholine; DPPC: 1,2-dipalmitoyl-sn-glycero-3-phosphocholine; DMG-PEG2K: 1,2-dimyristoyl-rac-glycero-3-methoxypolyethylene glycol-2000; DMPE-PEG2K: 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000];

3.3.2 Reprogramming proteins replacement

Stem cells with osteogenic differentiation capacity have great applications in engineering bone substitutes with biocompatible scaffolds for bone regeneration (Amini, Laurencin et al. 2012). Mesenchymal stem cells are good candidates due to their bispecific differentiation toward osteogenic lineages. However, their limited proliferation in elderly patients and multi-passage induced senescence limit their clinical applications (Jones and Yang 2011). On the contrary, iPSCs (induced pluripotent stem cells) derived from somatic cells, typically fibroblast reprogramming, have the potential to circumvent this obstacle, with a lower rate of iPSCs senescence after expansion, both *in vitro* and *in vivo*. [(Takahashi and Yamanaka 2006); (Takahashi, Tanabe et al. 2007)]. iPSCs showed osteogenic differentiation under stimulation of osteogenic factors (Teng, Liu et al. 2014); (Csobonyeiova, Polak et al. 2017).

Warren et al., reprogrammed fibroblasts to iPSCs by transfecting them with a mix of pseudouridine and 5-methylcytosine containing IVT mRNAs encoding reprogramming transcription factors which are KLF4/ c-MYC/ OCT4/ SOX2 (KMOS) or KLF4/ c-MYC/ OCT4/ SOX2/ LIN28 (KMOSL), respectively [(Warren, Manos et al. 2010)]. The KMOSL mRNAs mix induced ESC-like colonies almost ten days earlier than the KMOS

mRNAs mix, consistent with the previous report that LIN28 facilitated reprogramming [(Yu, Jin et al. 2017); (Hanna, Saha et al. 2009)].

3.4 Challenges and perspectives

Advancing the use of mRNA for bone regeneration requires an increase of cost-benefit value over traditional approaches such as rhBMP2 and pDNA. Although incorporation of modified nucleotides and HPLC purification of IVT mRNA improves its translation along with decreased activation of TLRs and PKR, the high cost remains a challenge. Optimization of the sequence of 5'-and 3'-UTR and ORF, is one reasonable approach to increase IVT mRNA stability and translation efficiency within cells [(Zhang, De La Vega et al. 2018)]. Asrani and colleagues provided an experimental framework for a high throughput screening assay to simultaneously evaluate hundreds of UTRs for optimized protein expression [(Asrani, Farelli et al. 2018)]. Translation efficiency enhancement resulting from sequence-engineering can even exceed nucleotides modification (pseudouridine) both *in vitro* and *in vivo* (Thess, Grund et al. 2015). However, to date, no universal UTRs can be translated across different genes and cell types.

Applying mRNA for bone regeneration will benefit from continuous efforts in RNA design particularly on circularization. Wesselhoeft et al. recently demonstrated that mRNA circularization yielded superior expression *in vivo* over linear mRNA, paralleled with decreased induction of TLRs (Wesselhoeft, Kowalski et al. 2019). Circularization of mRNA decreased TLR activation to the same extent as the inclusion of modified nucleotides in linear mRNA, demonstrating a new modality for therapeutic protein expression. A further advancement in the usage of mRNA for bone regeneration consists in sequence/structure optimization in osteogenic cells. Indeed, optimization of mRNA sequence has been mostly performed in dendritic cells or macrophages (Holtkamp, Kreiter et al. 2006); (Uchida, Kataoka et al. 2015); (Orlandini von Niessen, Poleganov et al. 2018), cancer cell lines (Trepotec, Aneja et al. 2019); (Wesselhoeft, Kowalski et al. 2018), fibroblasts (Hausburg, Na et al. 2015); (Ferizi, Aneja et al. 2016), epithelial cells (Andries, Mc Cafferty et al. 2015). But rarely on clinically-relevant human mesenchymal stem cells (Hausburg, Na et al. 2015). Based on the cell-type dependent effects of mRNA modifications on expression and immunogenicity (Uchida, Kataoka et al. 2015), incorporation of modified nucleotides should be tuned for MSCs.

Inspired by viruses impair host cells immune response, B18R protein, from Western vaccinia virus that binds to and neutralizes type I interferons, is frequently used as culture supplement after IVT mRNA transfection. (Alcamí, Symons et al. 2000); (Warren, Manos et al. 2010); (Yoshioka, Gros et al. 2013). Unlike B18R, Influenza A virus non-structural protein 1 (NS1) is a multiple functional protein blocking several steps in immune responses. Recent study showed that co-transfection of NS1 mRNA with

reporter mRNA, reduced immune response and increased reporter protein expression (Phua, Liu et al. 2017). NS1 from A/Texas/36/91 strain exhibited better performance than pseudouridine modification.

Transient expression is the inherent property of mRNA. The one-shot high expression is sufficient for vaccination, but sometimes not for protein-replacement therapies. For instance, to reconstruct bone nonunion continuous expression of osteogenic factors is needed. Entrapped mRNA complexes into a scaffold, sustained release of mRNA complexes resulting in cell multi-transfection and extended protein expression period. Hydrogels have been employed *in vitro* and *in vivo* as a therapeutic carrier to promote tissue formation. NIH3T3 cells encapsulated in fibrin hydrogel with 1 μ g pDNA coding firefly luciferase (fluc) expressed fluc for ten days with less fluc decrease in first 6 days (des Rieux, Shikanov et al. 2009).

Alternatively, Yoshioka et al. synthesized a self-replicating mRNA (RepRNA) for long-term multiple protein expression (Yoshioka, Gros et al. 2013). The proteins expressed from the RepRNA lasted for two weeks by a single-dose transfection. The polycistronic strategy suitable for bone regeneration as well, as several factors work synergistically in this process (Hankenson, Gagne et al. 2015).

4 Conclusion

In this review we described applications of mRNA and siRNA for bone regeneration. While the preclinical efficacy of RNA for bone regeneration has been demonstrated (Balmayor, Geiger et al. 2017); (Ghadakzadeh, Mekhail et al. 2016), the road to clinical evaluation requires evaluation in non-Human Primates. Bone is one of the dynamic tissues that has to support the body. Therefore, it involves many signaling pathways that have to be taken into account when building the therapeutic strategies. It is still highly challenging to restore large bone defect or nonunion fractures. The estimation is about one million of procedures that are occurring every year in Europe and the market estimated around € 5 billion. Progresses of RNA for bone regeneration will surely involve a combination of mRNAs encoding osteoinductive proteins with siRNAs targeting critical regulators of bone formation to maximize fracture resorption. The success of bone regeneration is highly dependent on osteogenic cells, their microenvironment and the mechanical and structural properties of the extracellular matrix. The selection of the right 3D scaffold is crucial to restore and maintain the regeneration process. These last years, 3D-printed templates have been proposed to fabricate patient-specific. Their preparation process is claimed to be easy, reproducible and can have specific micro-architecture with complex configuration (Zhang, Yang et al. 2019). However, the challenge is to get the right physical and biological properties and degradation, which must be in adequation with bone defect size and site. Hybrid 3D scaffold made with

bioactive and inorganic materials appeared to have good-compromised features. Incorporation RNA therapeutics in such scaffold will allow to create a patient specific bone graft on demand.

Acknowledgment

This work is supported by CNRS and University of Orléans. WANG Pinpin thanks the financial support from China Scholarship Council.

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