Therapeutic potential of nucleic acid aptamers against sclerostin in the treatment of osteoporosis

Quanxia Lyu

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DATE: August 21, 2017

STUDENT'S NAME: LYU Quanxia

THESIS TITLE: Therapeutic Potential of Nucleic Acid Aptamers against Sclerostin in the Treatment of Osteoporosis

This is to certify that the above student's thesis has been examined by the following panel members and has received full approval for acceptance in partial fulfillment of the requirements for the degree of Master of Philosophy.

Chairman: Prof. Yu Zhiling
Professor, Chinese Medicine - Teaching and Research Division, HKBU
(Designated by Dean of School of Chinese Medicine)

Internal Members: Dr. Yang Zhijun
Associate Professor, Chinese Medicine - Teaching and Research Division, HKBU
(Designated by Director of Chinese Medicine - Teaching and Research Division)

Prof. Zhang Ge
Professor, Chinese Medicine - Teaching and Research Division, HKBU

External Members: Prof. Xu Hongxi
Professor and Dean
School of Pharmacy
Shanghai University of Traditional Chinese Medicine

Issued by Graduate School, HKBU
Therapeutic Potential of Nucleic Acid Aptamers against Sclerostin in the Treatment of Osteoporosis

LYU Quanxia

A thesis submitted in partial fulfillment of the requirements for the degree of
Master of Philosophy

Principal Supervisor:
Prof. ZHANG Ge (Hong Kong Baptist University)

August 2017
DECLARATION

I hereby declare that this thesis represents my own work which has been done after registration for the degree of MPhil at Hong Kong Baptist University, and has not been previously included in a thesis or dissertation submitted to this or any other institution for a degree, diploma or other qualifications.

I have read the University’s current research ethics guidelines, and accept responsibility for the conduct of the procedures in accordance with the University’s Committee on the Use of Human & Animal Subjects in Teaching and Research (HASC). I have attempted to identify all the risks related to this research that may arise in conducting this research, obtained the relevant ethical and safety approval, and acknowledged my obligations and the rights of the participants.

Signature: Lyu Quanxia

Date: August 2017
Abstract

Osteoporosis is a skeletal disease characterized with poor bone quality and low bone mineral density. The pathogenesis of osteoporosis is the imbalance of bone resorption and bone formation. Two strategies can be employed to cure osteoporosis. One is to inhibit bone resorption and the other is to stimulate bone formation. Currently, therapeutic drugs approved by FDA are mainly antiresorptive agents. Till now, there is only one bone anabolic agent approved. Obviously, more efforts should be poured into the development of bone anabolic agents.

Sclerostin is a key negative regulator of osteoblast Wnt signaling making it a promising therapeutic target for bone anabolic therapy. Anti-sclerostin humanized monoclonal antibody romosozumab, which could effectively promote bone formation, has been accepted by the FDA for the review of biologic license application in 2017. However, there are several concerns about the humanized anti-sclerostin antibody, including immunogenicity, high cost of production and relative low stability.

Nucleic acid aptamers are short single stranded oligonucleotides. They can bind to their targets with similar high affinity as antibodies. Moreover, aptamers have some superior advantages compared to antibodies, such as no immunogenicity, easily synthesized, and high stability. Aptamers against sclerostin could be a promising alternative to antibodies in terms of promotion of bone formation and reversal of osteoporosis.

In this thesis, 20 rounds of SELEX were performed to select aptamers with high binding affinity and specificity to sclerostin. The inhibition potency of aptamer candidates to the antagonistic effect of sclerostin on Wnt signaling was also evaluated. Low $K_D$ and $EC_{50}$ values of aptamer candidates against sclerostin implied a great potential of sclerostin aptamer being the novel agents to promote bone formation. The study establishes the foundation for the next stage of preclinical studies and it will benefit the development of novel bone anabolic agents to reverse osteoporosis.

**Key words:** osteoporosis; bone anabolic agents; sclerostin; Wnt signaling; romosozumab; aptamer; affinity; specificity; inhibition potency.
Acknowledgements

I have an unforgettable experience in the journey to pursue the degree in Hong Kong Baptist University during the past two years. The courses provided by HKBU and its partner University are very useful for me to gain better understanding of my research. Besides, study resources in HKBU are very abundant and I really enjoyed the study atmosphere in the central library of HKBU. In addition, there are diverse experimental instruments of high tech available for students to use. Also, the gym in HKBU provides various equipment for us to release study stress and keep fit. Most of all, I got acquainted with so many excellent people in HKBU. Taking this opportunity, I would like to express my heartfelt thanks to them.

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I also wish to express my earnest gratitude to Dr. Yuanyuan Yu who gives me the biggest support both in this research thesis and in my daily life. She is not only a technique tutor of mine but also a bosom friend to me. The design of this thesis about therapeutic nucleic acid aptamers for the treatment of osteoporosis is greatly attributed to her wisdom. Every detailed experimental skill in molecular biology I mastered is patiently taught by her. It is her who makes the transition from analytical chemistry to drug discovery much easier for me to adapt. Without
her guidance, hardly can this thesis be completed successfully. Words are limited, but earnest gratitude for her is not. All the best wishes to her.

Special thanks should be delivered to Professor Chan Wing Hong who is the chairman of the program which I study in and my course instructor in organic chemistry. He is also an emeritus professor in HKBU and enjoys a high reputation in organic chemistry. When we were assigned to Haimen, Mainland of China, he left his family and travelled from HK to Haimen once a month in order to tutor us in courses as well as in experiments. Though life in Haimen was quite dull, he organized activities for us to get together and treated us with delicious foods. Every time when he stayed in Haimen, I really felt warm from bottom of my heart even in cold winter. May you be healthy and enjoy a happy life.

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Thank all of my labmates and classmates. It is a great pleasure for me to study and work with you together.

Last but not least, I would like to thank my parents for their endless love and care. You are always there backing me up for everything. Thank you for your thorough understanding allowing me to pursue my dreams on my own will. I also feel like expressing my gratitude to my elder brother and sister in law. Thank you for taking care of our parents.

Completion of this thesis may indicate an end of this stage of my study, but it is also a brand new start of the next stage of my life. All the best to us.
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<tr>
<td>BMD</td>
<td>Bone Mineral Density</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone Morphogenetic Protein</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>ELONA</td>
<td>Enzyme Linked Oligonucleotide Assay</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
</tr>
<tr>
<td>HRP</td>
<td>Horse Radish Peroxidase</td>
</tr>
<tr>
<td>LRP 5/6</td>
<td>Low Density Lipoprotein Receptor Related Proteins 5/6</td>
</tr>
<tr>
<td>OPG</td>
<td>Osteoprotegerin</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase Chain Reaction</td>
</tr>
<tr>
<td>PTH</td>
<td>Parathyroid Hormone</td>
</tr>
<tr>
<td>RANKL</td>
<td>Receptor Activator of Nuclear Factor Kappa-B Ligand</td>
</tr>
<tr>
<td>RANK</td>
<td>Receptor Activator of Nuclear Factor Kappa-B</td>
</tr>
<tr>
<td>SELEX</td>
<td>Systematic Evaluation of Ligand by Exponential Enrichment</td>
</tr>
<tr>
<td>SERM</td>
<td>Selective Estrogen Receptor Modulators</td>
</tr>
<tr>
<td>TMB</td>
<td>Tetramethylbenzidine</td>
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Chapter 1

Background and introduction
1.1 Overview of osteoporosis

1.1.1 Epidemiology of osteoporosis

Osteoporosis is a kind of skeletal disease which literally means porous bone. The World Health Organization (WHO) ranks osteoporosis as the second common disease after cardiovascular disease as it threatens over 200 million individuals’ health globally [1]. Either of the two criterions could be applied to define osteoporosis. One is low bone mineral density (BMD) with T-score no more than -2.5. The other is having a fragility fracture history [2]. Patients with osteoporosis typically show decreased bone strength, reduced bone mineral density and increased risk of fractures. It is estimated that one in three women and one in five men may suffer from osteoporotic fracture during their lifetime [3,4]. Fractures, especially hip and spine fractures, may cause serious effect to patients, such as long-term disability, loss of ability to take care of themselves and even death, let alone significant high health care costs [5,6].

Osteoporosis can be classified into two types, one is primary (including primary type 1 and type 2) osteoporosis and the other is secondary osteoporosis [7]. The osteoporosis in postmenopausal women (between age 50-70) is considered as primary type 1. The major reason for it may be the dramatic loss of estrogen after menopause, so primary type 1 osteoporosis is also called postmenopausal osteoporosis. Primary type 2 can be found both in males and females over age 70 which may be caused by age-related bone loss. Therefore, it
is also known as senile osteoporosis affecting both trabecular and cortical bone. For primary osteoporosis in men less than 70 years, the cause is unknown. So it can be called idiopathic osteoporosis. Secondary osteoporosis is common equally in men and women at any age. It has traceable etiology and may be resulted from many reasons, including nutritional factors, certain lifestyle behaviors, medications or diseases [8–10].

1.1.2 Regulation of bone metabolism

To better cure osteoporosis, it is essential to understanding the related bone biology in the occurrence of osteoporosis. Bone remodeling is a process which occurs throughout human’s life to renew bone and maintain bone mass and bone quality. Hence, the role of bone remodeling is critical to prevent bone micro-damage from accumulating which could eventually lead to fracture. In a healthy body, the rate of bone remodeling is relative low and there’s a homeostasis between bone formation and bone resorption. The bone mineral density is stable and bone strength is satisfied. However, when the rate of bone resorption is faster than that of bone formation, and this imbalance process maintains for a certain time, then osteoporosis may ultimately occur with measurable bone loss and skeletal fragility [11,12].

During the process, three types of bone cells are involved coordinately, including osteocytes (most numerous bone cells), osteoblast (to build bone cells) and osteoclast (to resorb bone cells). Normally, there is a ‘coupling effect’
between bone resorption and bone formation. That is to say the rate of these two components of bone remodeling may undergo synchronous change [13,14]. The remodeling cycle is comprised by the following sequential phases. First, osteoclasts are recruited and activated. Then bone resorption may last for 2-4 weeks followed by a reversal phase. When the resorption stops, bone formation takes place and lasts for 4-6 months. At last, mineralization process takes place to conclude the whole remodeling cycle [15,16].

Osteoclasts are bone-resorbing cells and they are originated from hematopoietic stem cells. The differentiation, activity and survival of osteoclasts is principally regulated by receptor activator of nuclear factor kappa-B ligand (RANKL). RANKL is mainly expressed by osteoblasts, osteocytes and other cells. When RANKL interacts with its receptor RANK, osteoclastic bone resorption will be promoted by inducted transcription factor and related enzymes. In another aspect, osteoblasts may produce osteoprotegerin (OPG) as a ‘decoy receptor’ to bind to RANKL and thereby the interaction between RANKL and RANK will be antagonized. Then, the osteoclasts mediated bone resorption can be inhibited [17]. Hence, the dynamic change of RANKL and OPG may greatly affect the magnitude of bone resorption. The expression of RANKL and OPG can be influenced by many factors, such as drugs, hormones, inflammatory cytokines and growth factors [18].
Osteoblasts are originated from mesenchymal stem cells in the skeleton and they are involved in the process of bone building. Osteoblastic bone formation is regulated by many molecular signals, including Wnt signaling proteins, human parathyroid hormone (PTH), PTH related peptides and so on. Among them, Wnt signaling proteins facilitated canonical Wnt/β catenin pathway is the principal stimulus for osteoblast differentiation. When Wnt signaling proteins bind to the low density lipoprotein receptor related proteins 5 and 6 (LRP 5/6) and Frizzled protein, the Wnt signaling pathway will be activated and stimulate the differentiation and mineralization of osteoblasts [14,19].

Osteocytes are the most abundant bone cells and function as ‘mechanosensors’ in response to gravity, physical activity and trauma. Osteocytes could initiate bone modeling process by releasing signaling proteins or by interactions between cells. Osteocytes can also express inhibitors of Wnt signaling proteins, such as sclerostin, to downregulate osteoblastic bone formation [12].

Based on these understandings of osteoporosis occurrence and regulators, two therapeutic strategies can be applied to cure osteoporosis. One is to slow down osteoclast-mediated bone resorption to prevent bone loss and the other is to speed up osteoblast-mediated bone formation.

**1.1.3 Therapeutic drugs for the treatment of osteoporosis**

A number of drugs for curing osteoporosis approved by the Food and Drug Administration (FDA) are designed based on the above therapeutic rationale.
Among them, the major ones are antiresorptive agents including four bisphosphonates (alendronate, risedronate, ibandronate and zoledronate), estrogen, raloxifene, calcitonin and denosumab [20,21]. Currently, the only approved bone anabolic drugs that stimulate bone formation to reverse osteoporosis is PTH hormone peptides, including PTH 1-34 fragment (teriparatide) which is available worldwide and PTH 1-84 whole molecule which is only available in many European countries but not commercialized any more [22]. Table 1.1 presents the current FDA approved drugs for the treatment of osteoporosis.

1.1.3.1 Antiresorptive agents

Bisphosphonates are commonly used anti-osteoporosis agents. They are pyrophosphate analogs and can be incorporated into the skeleton by strongly binding to the hydroxyapatite in the bone. Thereby, the osteoclast activity is reduced and bone resorption is inhibited [7]. The superior property of bisphosphonates is that it can produce ‘drug holiday effect’ to osteoporosis patients. That is, after discontinuing administration of bisphosphonates, they can still exert their effects to prevent bone resorption [23]. However, the poor compliance due to frequently administration limits their therapeutic efficacy [1]. Furthermore, many studies reported that long term use of bisphosphonates may associate with many risks, such as osteonecrosis of the jaw, atypical femur fractures and atrial fibrillation [20,23–25].
Postmenopausal osteoporosis is caused by a sudden deficiency of estrogen. So the idea to reverse this type of osteoporosis seems to be simple: to supplement estrogen. Estrogen replacement therapy was once the only FDA approved treatment targeted for postmenopausal osteoporosis. The mechanism of its function is likely to inhibit bone resorption by regulating many estrogen-dependent regulatory factors, such as increasing expression of OPG and decreasing the expression of RANK [26]. The beneficial effects of estrogen to conserve bone mass and prevent osteoporotic fracture is of no doubt. However, cumulative evidences demonstrating the increased risk of breast cancer, uterine cancer, stroke and cardiovascular disease greatly limit the extensive use of estrogen [7,27,28].

Raloxifene (brand name Evista) is a member of the selective estrogen receptor modulators (SERM), which act as estrogen antagonists to bind to estrogen receptor. Currently, it is the only approved SERM by FDA to prevent and treat postmenopausal osteoporosis. Though it may increase bone mineral density in the cases of vertebral fractures, decreased incidence of hip fractures or other non-vertebral fractures has not been shown [29]. It was also reported that the effect of raloxifene is not as good as that of bisphosphonates in the treatment of osteoporosis [1,30,31]. Moreover, a recent case report in 2016 pointed out that raloxifene, to some extend, stimulated the breast cancer growth [32].
Calcitonin, a peptide hormone with 32 amino acids, is mainly secreted by parafollicular cells of the thyroid gland. It plays an important role in mineral metabolism and bone homeostasis, acting as a physiologic endogenous inhibitor of bone resorption by inhibiting osteoclast activity. Though it is controversial whether there is an association between the use of calcitonin and malignancy, calcitonin was recently removed from the market in several countries and FDA also did not recommend long-term use of it for the treatment of osteoporosis [15,33].

Denosumab (brand name Prolia) is a monoclonal antibody against RANKL given subcutaneously twice a year. It is currently considered to be the most potent novel antiresorptive agent to prevent bone loss [20,34]. The function of desozumab is just like OPG mentioned above acting as a ‘decoy receptor’ for RANKL. It binds to RANKL with more superior specificity than OPG and RANK to inhibit the interaction of RANKL and RANK. Thereby, the differentiation and activation of osteoclasts is inhibited and so as the bone resorption mediated by osteoclasts [27]. However, RANKL is not only expressed by osteoblasts but also by immune cells, so the potential adverse effect by long term use of desozumab may be a major concern [20].

All together, each antiresorptive agent has its unique action mechanism and noticeable drug efficacy, as well as its unneglectable limitations in the treatment of osteoporosis. Due to the coupling effect between bone resorption and
bone formation, after about 6 months when antiresorptive agents decrease the rate of bone resorption, the rate of bone formation is also reduced along with it. Then the bone remodeling reaches at a new equilibrium with a lower rate. Overall, moderate augment of bone mineral density and bone mass can be achieved by these antiresorptive agents [35].

1.1.3.2 Bone anabolic agents

Teriparatide (brand name Forteo) as PTH peptides is the only approved bone anabolic agent. It has the capacity to bring promising results in increasing bone mineral density for curing osteoporosis. However, there are still some shortcomings for this kind of therapy. For example, many patients do not response to PTH peptides and for those who have a skeletal response, the drug efficiency wanes over time. Moreover, the use of PTH may also cause various side effects, such as hypercalcemia and hypercalciuria. Notably, high dose of PTH administration for a long term may lead to osteosarcoma. Because of this, Europe and US limit its use to 18 months and 24 months respectively [36–38].

Osteoanabolic agents have superior abilities to increase bone strength relatively to traditional antiresorptive agents [20,35]. Hence, more efforts should be made to develop additional anabolic agents for better treatment of osteoporosis.
# FDA approved drugs for the treatment of osteoporosis

<table>
<thead>
<tr>
<th>Classification</th>
<th>Drug</th>
<th>Company</th>
<th>Mechanism of action</th>
<th>Disadvantage</th>
<th>Administration</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Antiresorptive Drugs</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Bisphosphonates</strong></td>
<td>Alendronate</td>
<td>Merk</td>
<td>incorporate into skeleton by strongly binding to hydroxyapatite in bones</td>
<td>poor compliance due to frequently administration; long term use may cause many risks</td>
<td>oral</td>
</tr>
<tr>
<td></td>
<td>Risedronate</td>
<td>Takeda</td>
<td></td>
<td></td>
<td>oral</td>
</tr>
<tr>
<td></td>
<td>Ibandronate</td>
<td>Roche</td>
<td></td>
<td></td>
<td>oral/ intravenous</td>
</tr>
<tr>
<td></td>
<td>Zoledronate</td>
<td>Novartis</td>
<td></td>
<td></td>
<td>intravenous</td>
</tr>
<tr>
<td><strong>Estrogen Replacement Therapy</strong></td>
<td>Estrogen</td>
<td>not applicable</td>
<td>regulate estrogen-dependent regulatory factors, such as increasing OPG and decreasing RANK</td>
<td>increased risk of breast cancer, uterine cancer, stroke and cardiovascular disease</td>
<td>oral/ intravenous</td>
</tr>
<tr>
<td><strong>Selective Estrogen Receptor Modulators</strong></td>
<td>Raloxifene</td>
<td>Eli Lilly</td>
<td>act as an estrogen agonist in bone</td>
<td>Side effects: drug-related hot flashes and leg cramp;</td>
<td>oral</td>
</tr>
<tr>
<td><strong>Calcitonin</strong></td>
<td>Calcitonin</td>
<td>not applicable</td>
<td>inhibit osteoclast activity in bones protecting against calcium loss from skeleton</td>
<td>potential association with cancer</td>
<td>subcutaneous</td>
</tr>
<tr>
<td><strong>Humanized Monoclonal Antibody</strong></td>
<td>Denosumab</td>
<td>Amgen</td>
<td>block interaction between RANKL and RANK</td>
<td>potential adverse effect on immune cells</td>
<td>subcutaneous</td>
</tr>
<tr>
<td><strong>Anabolic Drugs</strong></td>
<td>PTH Peptides</td>
<td>Teriparatide</td>
<td>Eli Lilly</td>
<td>activate osteoblasts to increase bone formation</td>
<td>drug efficiency wanes over time; risk of osteosarcoma if long term use</td>
</tr>
</tbody>
</table>
1.1.4 Physiologic roles of sclerostin in bone formation

Sclerostin, encoded by SOST gene, is a secreted glycoprotein with sequence similar to the DNA family of bone morphogenetic protein (BMP). The role of sclerostin in bone formation came to light specifically by the studies of two rare genetic diseases. One is sclerosteosis, a disease characterized by progressive bone overgrowth due to the loss of function mutations in sclerostin. The other is van Buchem disease which is typical by its high levels of bone mineral density. This genetic disease is caused by a loss of downstream SOST gene encoding the sclerostin protein [35,39,40]. To further understand the association between sclerostin and bone formation, a study generated SOST knockout mice and found increased bone volume, bone mineral density and bone strength in the built SOST knockout mice [41]. These genetic studies in human and mice all show that the deficiency of sclerostin could result in increased bone density, indicating sclerostin is a key negative regulator of bone formation [42].

Ample evidence indicates that sclerostin could inhibit osteoblastic bone formation by downregulating Wnt signaling. And Wnt signaling has an indispensable role in bone morphogenetic protein (BMP) stimulated osteoblastic differentiation. Sclerostin binds to low density lipoprotein receptor-related protein 5 and 6 (LRP5/6) which are the receptors of Wnt signaling proteins [43–45]. As an antagonist of Wnt signaling proteins, sclerostin inhibits BMP stimulated bone
formation by antagonizing Wnt signaling rather than directly affect BMP signaling [46,47]. The simplified role of sclerostin can be seen in Figure 1.1.

Sclerostin is expressed almost exclusively in bone tissues, particularly by osteocytes [47,48]. Besides, patients with deficient sclerostin just show increased bone strength with no other abnormalities [49]. Therefore, sclerostin is a promising therapeutic target for the development of novel anabolic agents to treat with osteoporosis.

**Figure 1.1 Sclerostin antagonizes Wnt signaling.** Wnt ligands bind to LRP5/6 and seven transmembrane Frizzled co-receptors on osteoblast stimulating downstream cascades to increase bone formation. Sclerostin could antagonize Wnt signaling by binding to LRP5/6 and therefore inhibit bone formation. Adapted from two articles [14,19].
1.1.5 Antibodies against sclerostin for reversing osteoporosis

The antagonistic effect of sclerostin on Wnt signaling inspires us a new strategy in the treatment of osteoporosis: to block the binding of sclerostin to LRP 5/6 receptors by inhibition of sclerostin. By this way, Wnt signaling pathway will be reactivated and bone formation will be promoted. Based on this understanding, a series of monoclonal antibodies against sclerostin are under development.

1.1.5.1 Promising bone anabolic effect of anti-sclerostin antibodies

Sclerostin antibodies in animal models (ovariectomized female rats, aged male rats, mice and gonad-intact female cynomolgus monkeys) showed increased bone quality on the inhibition of sclerostin [45]. Two monoclonal antibodies neutralizing sclerostin were well developed in human studies including romosozumab (AMG 785, CDP7851, Amgen) and blosozumab (Eli Lilly). Completed Phase I and phase II studies for blosozumab and romosozumab showed noticeable osteoanabolic effects in subjects.

Phase III clinical trials evaluating the safety and efficacy of romosozumab for osteoporosis are currently ongoing [14,45,50]. In these trials, 7180 postmenopausal women with osteoporosis at total hip or femoral neck were enrolled in the fracture study in postmenopausal women with osteoporosis (FRAME). They were randomly subcutaneously injected of romosozumab or placebo monthly for 12 months. Thereafter, all patients were administrated with denosumab every 6 months for 12 months as a transition. The result showed that
at 12 months, compared with patients in the placebo group, patients received romosozumab were associated with a lower risk of vertebral fracture. And at 24 months after the denosumab treatment, the rates of vertebral fracture were significantly lower in patients received romosozumab than those received placebo. As for the adverse events, such as instances of hyperostosis, cardiovascular events and cancer, they were almost balanced in these two groups [51].

Based on these encouraging results, FDA has recently accepted to review the biologics license application (BLA) for romosozumab and has set a target action date for Prescription Drug User Fee Act on July 19, 2017.

1.1.5.2 Limitations of antibody drugs

Though romosozumab against sclerostin shows encouraging results in postmenopausal women with osteoporosis, there are still several concerns for the antibodies as the most widely used protein-binding reagent. One concern is adverse effects caused by immune response to antibodies. Clinical trials on romosozumab reported that neutralizing anti-romosozumab antibodies were developed in several patients, indicating the possibility of drug resistance for long term treatment due to the immunogenicity of antibodies [52,53]. And one subject discontinued the clinical study due to pruritus related with the study medication [52]. Besides, according to the results of phase III study which were reported in NEJM in 2016, one atypical femoral fracture and two cases of jaw osteonecrosis were observed in patients treated with romosozumab [51]. Another concern is
poor reproducibility. There is a batch to batch variability in the production of antibodies making it hard to reproduce the original results. Furthermore, the production could be laborious and of high cost with high risk of failure [54–56]. Relatively low stability is another concern of antibodies as drugs. Antibodies are made from proteins and can be easily denatured resulting in limited shelf life. The handing process, transportation and storage conditions of antibodies should be treated cautiously [57,58].

Therefore, efforts should be tried to find additional anti-sclerostin agents as bone anabolic drugs. They are expected to have the same superior affinity and specificity relative to antibodies, but with no immunogenicity. The additional bone anabolic agents should also be easily produced with high stability of low cost. It seems that therapeutic oligonucleotide aptamers could be the option to reverse osteoporosis.

1.2 Overview of nucleic acid aptamers

1.2.1 Development of nucleic acid aptamers against sclerostin

Nucleic acid aptamers are short single stranded oligonucleotides usually with 20-70 bases. The term aptamer is originated from a Latin word ‘aptus’ which means ‘to fit’ indicating a lock and key relationship between aptamers and their targets. That is to say, aptamers have the capacity to bind to their targets with robust affinity and specificity through the interactions of three-dimensional
conformational complementarity. The targets for aptamers are mainly proteins, virus, living cells, nucleotides and amino acids [57–60].

Due to the satisfactory binding affinity and specificity which is similar to antibodies, aptamers could be considered as the alternative anti-sclerostin agent. Moreover, there are several important advantages for aptamers as alternative agent relative to antibodies. First, the small size makes it impossible for aptamer to be recognized by immune system. Then the immunogenicity caused by aptamers will be minimal [61]. Second, aptamers can be chemically synthesized in vitro making the production of aptamers less expensive and less risky [62]. Furthermore, aptamers are more stable than antibodies with longer shelf life. They are temperature resistant and tolerate various conditions for transportation without special treatments such as continuous cold chain [57,58].

1.2.2 Therapeutic aptamers in clinical studies

Currently, there are 11 aptamer drugs under different stages of clinical evaluations for the treatment of macular degeneration, cancer, coagulation and inflammation [63,64]. Of note, one of them against vascular endothelial growth factor (VEGF), named pegaptanib (Macugen), was approved by FDA in 2004 for the treatment of age-related macular degeneration [58,65]. These previous successful practices strengthen our confidence in disease therapy by therapeutic aptamers. All together, anti-sclerostin aptamers could be promising bone anabolic agents alternative to anti-sclerostin antibodies for the treatment of osteoporosis.
1.2.3 Systematic Evolution of Ligand by Exponential Enrichment (SELEX)

Aptamers can be obtained through the method of SELEX. In 1990, two labs (the Gold lab and the Szostak lab) performed the method of SELEX to select aptamers independently [59,60]. There are several steps in the conventional SELEX method. First of all, a screening library containing billions of random single stranded oligonucleotides (taking ssDNA for example) should be chemically synthesized. The structure of the random oligonucleotides mainly has two parts. One is the conserved sequences at both ends which are used for primer binding in polymerase chain reaction (PCR). The other is a random sequence in the middle of the two conserved sequences. The random sequence is generally consisted of 20-40 bases which makes the size of the screening pool as large as $10^{12}$-$10^{15}$ and guarantees the high diversity of the library. Next, the target proteins are incubated with the random aptamer pool. During a partition process, some sequences bind to the target proteins and the others do not bind. Then the bound sequences are separated from those unbound through proper separation methods. The unbound sequences are washed off, whereas the bound ones are eluted and then amplified by PCR. After preparation of single stranded oligonucleotides, one cycle of positive selection is completed. Sometime, negative or counter selection is needed to exclude sequences that can bind both to target proteins and unwanted proteins. Unwanted proteins are added to the above eluted bound sequences to
incubate. For this time, the bound sequences to unwanted proteins are washed off and those unbound are collected. Through this way, the sequences only bound to target proteins are remaining. The whole selection process is usually made up of 7-20 cycles, repeating the process of incubation, partitioning, elution and amplification, until sequences with high binding affinity are obtained. The obtained aptamer candidates are then transformed into bacteria for sequencing and subsequent characterization [57-59]. The detailed procedures of the conventional SELEX can also be seen in Figure 1.2 [57,65].

Figure 1.2  Scheme of conventional SELEX procedure. Aptamers of different sequences are represented in different colors. Adapted from two works contributed by Yu and Barman [57,65].
1.3 Research purpose and workflow

Based on the overviews of therapeutic drugs for osteoporosis and aptamers as potent alternative drugs, the purpose of the research is to identify anti-sclerostin aptamers. The desired aptamer candidates should have high binding affinity and specificity to sclerostin, as well as noticeable inhibition potency to sclerostin. As is mentioned above, sclerostin could exert an antagonistic effect on Wnt signaling. Hence, the inhibition of sclerostin could result in reactivation of Wnt signaling and further stimulation of bone formation. This study will definitely benefit the next stage preclinical studies, as well as the development of novel osteoanabolic drugs to reverse established osteoporosis. To realize this purpose, three sub-aims are set as follows:

1. Selection of aptamer candidates by using established SELEX method.

2. Assessment of the bind affinity and specificity of aptamer candidates to sclerostin by using an aptamer enzyme-linked oligonucleotide assay (ELONA).

3. Evaluation of inhibition potency of aptamer candidates to the antagonistic effect of sclerostin on Wnt signaling in osteoblast MC3T3-E1 cells by using a TOP-Wnt-induced luciferase reporter assay.

The workflow of this thesis is shown in Figure 1.3.
Figure 1.3  **Workflow of the study.** First, identification of aptamer candidates against sclerostin using SELEX. Next, characterization of selected aptamer in terms of binding affinity and specificity. Finally, evaluation of inhibition potency of the aptamer candidates with high binding affinity and specificity to sclerostin in MC3T3-E1 cells.
Chapter 2

Identification of nucleic acid aptamers against sclerostin
2.1 Aims and overview

In this chapter, selection of aptamer against human recombinant sclerostin was conducted using SELEX. 20 rounds of positive selection and 5 rounds of counter selection (NTA beads in 3 rounds and bovine serum albumin (BSA) in other 2 rounds) were performed in this SELEX section. Aptamers selected from positive selection and counter selection were sequenced.

The strategy to prepare single strand DNA in this study is based on magnetic beads. Figure 2.1 presents the scheme to prepare ssDNA.

![Figure 2.1 Schematic illustration of ssDNA separation using magnetic separation.](image)

First, the random ssDNA library was applied on PCR with PCR reagents containing biotinylated reverse primer. After PCR reaction, pre-conditioned streptavidin-coated beads were added to the dsDNA with one strand containing biotinylated end which was produced by PCR. Then, a magnetic
rack was applied to the system which resulted in the attraction of one strand with the mixture of biotin and streptavidin to the magnetic rack. Finally, under the effect of added alkaline, the desired ssDNA was produced. Adapted from Liang’s work [67].

2.2 Experimental section

2.2.1 Experimental materials

SELEX ssDNA library

ssDNA library was synthesized by company of Invitrogen. The random sequence in the library was consisted of two conserved ends with each 18 nts and central random region with 40 nts. Based on the designed conserved sequences, forward primer and reverse primer with and without biotin label were all synthesized. The sequence in total (76 nts) is:

CGTACGGTCGACGCTAGC(N)₄₀CACGTGGAGCTCGGATCC

Recombinant human sclerostin

Recombinant human sclerostin (Catalog # 48) used in this study is purchased from the company of Novoprotein. It is produced by mammalian expression system.

Magnetic beads

NTA magnetic beads were purchased from Qiagen which were used to immobilize target sclerostin. Dynabeads MyOne Streptavidin T1 beads were
purchased from Invitrogen which were used for preparation of single-stranded DNA aptamers.

*Reagents in PCR reaction*

Mg$_2$SO$_4$, dNTPs, 10 × pfx buffer and pfx proofreading polymerase were all purchased from Invitrogen.

*Reagents used in ELONA*

Lambda (λ) exonuclease was from New England Biolabs. 3,3′,5,5′-tetramethylbenzidine (TMB) liquid substrate system was from Sigma. And streptavidin-horse radish peroxidase (HRP) conjugate was from Invitrogen.

*Other materials*

1 kp plus DNA ladder, 100 bp DNA ladder and TA cloning kit were all purchased from Invitrogen. QIAEX II gel purification kit was purchased from Qiagen.

**2.2.2 Preparation of buffers and solutions**

In SELEX experiment, PBS buffer containing 1 mM MgCl$_2$, 20 mM imidazole and 0.05% Tween 20 was used as binding and washing buffer for protein and DNA. 100 mM Tris-HCl in pH 7.5, 1 mM EDTA and 2 M NaCl were prepared for streptavidin as binding and washing buffer. 0.1 M NaOH was used as alkaline buffer in separation of single-stranded DNA. 50 mM Tris pH 7.5 was used as buffer to recover single-stranded DNA.
In ELONA assay, blocking buffer was made up of PBS, 0.1% Tween 20 and 1% BSA. Washing buffer was also composed of PBS, 0.1% Tween 20 and 1% BSA. Strep-HRP binding buffer was diluted 1:10000 in washing buffer. And 2 M H$_2$SO$_4$ was used as buffer to terminate the reaction.

### 2.2.3 Procedures in SELEX

#### 2.2.3.1 Immobilization of sclerostin on NTA beads

First, NTA magnetic beads were washed three times using 500 µL protein binding and washing buffer. Then the supernatant was carefully removed after the solution system containing beads was applied to a magnet. Subsequently, the NTA beads were incubated with appropriate amount of sclerostin at 4 °C for 1 hour on a rotator under a gentle speed. Next, the solution system containing beads together with sclerostin were applied to the magnet. Then, the supernatant containing unbound sclerostin was pipetted out and the bound sclerostin to beads were remaining in the vial. To remove the unbound sclerostin thoroughly, the beads-sclerostin complex was washed for three times using 500 µL protein binding and washing. Finally, through these steps, sclerostin was immobilized onto the NTA beads waiting for the next stage usage.

#### 2.2.3.2 Incubation of ssDNA library and beads-sclerostin complex

500 µL DNA binding and washing buffer was used to wash the beads-sclerostin complex for three times. Then 1 mL DNA binding and washing buffer were added to the complex in a vial. In order to keep the ssDNA staying in
simple and unified structure, the library was heated to 95 °C for 5 min and rapidly cooled down to 4 °C. Then the library was incubated with the beads-sclerostin complex at room temperature on a roller for 30 minutes. Next, the beads-sclerostin-ssDNA complex was applied to the magnet to separate the complex from the solution system. The supernatant which contained the free ssDNA was pipetted out. Subsequently, the beads-sclerostin-ssDNA complex was washed for three times with 500 µL DNA binding and washing buffer to remove the non-specific binding. The complex was pre-washed with 500 µL dd H2O and 0.05% Tween 20 for two times and then the complex was resuspended with 500 µL buffer of dd H2O and 0.05% Tween 20.

2.2.3.3 Amplification of the bound ssDNA to beads-sclerostin complex

The above complex was transferred to PCR tubes which were added in 500 µL dd H2O and 0.05% Tween 20. Table 2.1 presents the materials used in the PCR reaction.

Table 2.1 Materials added in PCR

<table>
<thead>
<tr>
<th></th>
<th>Volume (µL)</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Template</td>
<td>10</td>
<td>-</td>
</tr>
<tr>
<td>pfX polymerase</td>
<td>0.5</td>
<td>1.25 U</td>
</tr>
<tr>
<td>Forward primer</td>
<td>10</td>
<td>1 µM</td>
</tr>
<tr>
<td>Reverse primer (with biotinylated end)</td>
<td>10</td>
<td>1 µM</td>
</tr>
<tr>
<td>dNTP</td>
<td>1</td>
<td>0.1 mM</td>
</tr>
<tr>
<td>MgSO4</td>
<td>1</td>
<td>0.5 mM</td>
</tr>
<tr>
<td>10 × enhancer</td>
<td>1</td>
<td>0.1×</td>
</tr>
<tr>
<td>10 × buffer</td>
<td>10</td>
<td>1×</td>
</tr>
<tr>
<td>H2O</td>
<td>56.5</td>
<td>-</td>
</tr>
<tr>
<td>Total</td>
<td>100</td>
<td>-</td>
</tr>
</tbody>
</table>
A series of experiments was carried out to find the optimal condition for PCR reaction, especially for the effective PCR cycle number. The PCR cycle numbers were set as 8, 10, 12, 14, 16 and 18 in the amplification of ssDNA library.

2.2.3.4 Preparation of single strand DNA

When the PCR amplification was finished, the 100 µL PCR product was applied to the magnet. After an equilibrium was reached, the supernatant containing the dsDNA produced in PCR reaction was pipetted to a new vial of 1.5 mL. The 100 µL streptavidin beads were washed with 500 µL 1 × streptavidin binding and washing buffer for three times. Then the beads were suspended using 100 µL 2 × streptavidin binding and washing buffer. Next, the washed beads with dsDNA were incubated for 15 minutes on a roller at room temperature. One strand of dsDNA with biotinylated end would bind with the streptavidin modified beads. Proper time was given to allow the binding reach an equilibrium. Then the complex of beads and dsDNA was washed with 500 µL 1 × streptavidin binding and washing buffer for three times in order to remove nonspecific binding.

Next, 50 µL 0.1 M NaOH alkaline solution was added into beads-dsDNA complex for 5 minutes at room temperature to denature the dsDNA by breaking the hydrogen bonds between two strands of dsDNA. Then, the system was applied to the magnet to separate beads from solution. The biotinylated strand bound to streptavidin beads would attach to the magnet, while the other strand would go
freely into the solution system. The eluent containing ssDNA was collected and transferred to 100 µL recovery buffer. Finally, the selected ssDNA was stored at -20 °C until use. Till now, one round of positive selection of ssDNA was completed.

After each round of selection, PCR was checked with 4% agarose gel to confirm the presence of ssDNA in the obtained secondary library.

The whole selection section was performed with 20 rounds of positive selection and 5 rounds of counter selection. The 5 rounds of counter selection were set to increase binding specificity of aptamers to sclerostin. Among the 5 counter selection rounds, NTA beads was used in 3 rounds of counter selection in order to exclude ssDNA which has relative strong background binding capacity to beads. BSA was used as undesired protein in the other 2 rounds of counter selection in order to exclude ssDNA that bound both with sclerostin and BSA.

The incubation time in each round was set as 30 minutes. However, the amount of sclerostin used in each round was different. Overall, the variety of the ssDNA in library would be less and less along with the increase of selection rounds. For the first three positive rounds, 200 pmol sclerostin was used. For round 4-6, 100 pmol sclerostin was used. The next three round 7-9 was 75 pmol sclerostin. For round 10-14, 50 pmol sclerostin was used. Then for the remaining round of 15-20, the amount of sclerostin used was 30 pmol.
2.2.4 Enzyme linked oligonucleotide assay (ELONA)

ELONA was applied to determine the binding affinity of aptamers to sclerostin. The mechanism of ELONA is very similar to that of ELISA [68]. Single-stranded DNA with biotin should be generated first. To realize this aim, lambda (λ) exonuclease was used because it could cleave base one by one upon the recognition of phosphorylated terminal.

The detailed procedure was as follows. First, PCR was performed to produce dsDNA with biotinylated forward primer and phosphorylated reverse primer. The PCR product was checked on 4% agarose gel and the target bands were purified by QIAEX II gel purification kit. Nanodrop was used to measure the concentration of the purified dsDNA. Then the purified dsDNA was digested using λ exonuclease (10 units λ exonuclease with 1 µg dsDNA) at 37 °C for 30 minutes. This reaction could be terminated by incubation at 75 °C for 10 minutes. Then the generated ssDNA was purified and its concentration was also measured. Next, the prepared biotinylated ssDNA were recovered in recover buffer. They were denatured at 95 °C for 5 minutes and then they were rapidly cooled down at 4 °C for 10 minutes. At last, the biotinylated ssDNA was stored in room temperature until use. The strategy to prepare the biotinylated ssDNA is shown in Figure 2.2.
Figure 2.2 Strategy to prepare the biotinylated aptamer by using λ exonuclease.

The mechanism of the ELONA assay which is quite similar to that of ELISA is presented in Figure 2.3. The detailed procedure was as follows. 160 ng sclerostin was pipetted to be coated onto a 96-well microtiter plate in 100 µL protein binding and washing buffer. Sclerostin was incubated in the plates at 4 °C overnight. Blocking buffer was used to block the plate for 1 hour at room temperature and the blocked plate was washed with protein binding and washing buffer for four times.

Appropriate amount of biotinylated aptamers was added into each well respectively together with DNA binding and washing buffer (the final volume of aptamers was 100 µL). The biotinylated aptamers were from round 15, round 20 and counter 5. The biotinylated aptamers were incubated with different concentrations of coated sclerostin for 45 minuets at room temperature on a shaker. After incubation, the plate was washed with DNA binding and washing
buffer for four times in order to remove the non-specific binding with coated sclerostin or plate surface. Then the plate was washed with PBST+0.1% BSA four times to pre-condition the plate.

100 µL streptavidin-HRP (1:10000 dilute by PBST+0.1% BSA) was added into each well and was incubated for 30 minutes. After incubation, the plate was washed for four times using PBST+0.1% BSA.

50 µL TMB (substrate) was added to each well and incubated for 20 minutes. The reaction of substrate and HRP would give out colored product. Then 50 µL 2 M H₂SO₄ was added to terminate the reaction. Finally, absorbance of the colored product at 450 nm would be measured with microplate reader.

Figure 2.3 Mechanism of ELONA assay.
2.3 Results and discussions

2.3.1 Identification of optimal PCR cycle number

In order to get the optimal amplification of ssDNA library, a series of PCR reaction was performed in which the cycle numbers were set as 8, 10, 12, 14, 16 and 18. Figure 2.4 shows the amplification results of ssDNA in each PCR reaction with different cycle numbers. According to the figure, there were noticeable side products in PCR reaction with 8, 10, 12 and 14 cycle numbers. For PCR results with cycle numbers of 16 and 18, the band was singular and clear. Considering time and energy saving, optimal PCR cycle number was set at 16 for the following PCR reactions in this study.

![Figure 2.4 Identification of optimal PCR cycle number.](image)

2.3.2 Identification of aptamers against sclerostin

20 rounds of positive selection and 5 rounds of counter selection were performed to identify the aptamers against sclerostin. Among the 5 rounds of
counter selection, 3 rounds were performed by using NTA beads to exclude aptamers bound to beads. 2 rounds were also performed using BSA protein to increase binding specificity of aptamers to sclerostin.

ssDNA with expected size of 76 nts were checked in agarose gel. Bound ssDNA in each round were clearly shown on the gel, which indicated that aptamers against sclerostin-beads were successfully identified and amplified.

2.3.3 Enrichment of ssDNA with high binding affinity

Binding affinity of ssDNA pool against sclerostin was evaluated by ELNOA assay in which all data were triplicated. Figure 2.5 presents the curves and corresponding $K_D$ values of ssDNA pool in the original library, library of round 10 and library of round 20.

Aptamers identified in round 10 and 20 had relatively low $K_D$ values (20 nM and 50 nM respectively). This nanomolar level of $K_D$ values indicated a successful selection of aptamers with high binding affinity to sclerostin. The aptamer pool in round 20 had a higher binding affinity to sclerostin than in round 15 implying aptamers of higher affinity were enriched in round 20 through SELEX.
Figure 2.5 Binding affinity of aptamers from selection round of 0, 15 and 20 by ELONA assay.

2.4 Summary of chapter 2

In this chapter, 20 rounds of SELEX were performed to select aptamers against sclerostin with high binding affinity. Total 5 rounds of counter selection were conducted to increase the binding specificity of selected aptamers. Among them, 3 rounds of counter selection were used NTA beads to exclude aptamers bound to beads, and 2 rounds of counter selection were used BSA as the unwanted protein to exclude aptamers bound to BSA.

Bound ssDNA in each round of selection were monitored by agarose gel. It showed that the desired aptamer with expected size of 76 nts were successfully identified and amplified in the final round of selection.
ELONA assay was performed to evaluate the binding affinity of original ssDNA library and aptamer pools in round 10 and round 20. It showed quite low $K_D$ of aptamer pool against sclerostin in round 10 and round 20. And the highest binding affinity in round 20 indicated the successful enrichment of aptamers with high binding affinity. Therefore, the SELEX performed in this study was feasible to select aptamers with high binding affinity.
Chapter 3

Characterization of selected ssDNA aptamers against sclerostin
3.1 Aims and overview

In this chapter, aptamers selected from last chapter were sequenced and synthesized for further use. Then the synthesized aptamers were characterized in aspect of binding specificity and binding affinity.

3.2 Experimental section

3.2.1 Experimental materials

Selected aptamers (aptscl 1/2/3/5/6/9/11/13/15/17/20/21/23/27/28/31/34/37/46/47/50/51/106/68/72/62/29/118/105/132/82/122/66/86/36/67/140/114/76/91/69/136/37/107/137/56/50, in total 48 aptamer candidates) with high frequency of occurrence in aptamer pool of round 20 were sequenced by next generation sequencing. They were all synthesized and modified with N-terminal biotin by Integrated DNA Technologies (IDT). TA cloning kit was from Invitrogen.

3.2.2 Buffers and solutions

Protein binding and washing buffer, DNA binding and washing buffer, ELONA blocking buffer and washing buffer and strep-HRP binding buffer were all the same as in chapter 2.

3.2.3 Assessment of the binding specificity

Table 3.1 shows the strategy to assess binding specificity of 48 aptamer candidates and one random sequence for control to sclerostin. 1 μM of each aptamer candidate and random sequence were used to determine the binding
specificity to sclerostin and to BSA by method of ELONA. The detailed procedure of ELONA was as that in chapter 2. Of note, 48 aptamer candidates and one random sequence were added both to sclerostin coated plate and BSA coated plate. Then the comparison of binding affinity of aptamers to sclerostin and to BSA could be obtained.

Table 3.1 Strategy to assess binding specificity

<table>
<thead>
<tr>
<th>Group (n=3)</th>
<th>Protein</th>
<th>Concentration</th>
<th>Method</th>
<th>Evaluation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1-48: one aptamer in each group</td>
<td>Sclerostin</td>
<td>1 µM</td>
<td>ELONA</td>
<td>Absorbance at 450 nm</td>
</tr>
<tr>
<td></td>
<td>BSA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 49: one random sequence</td>
<td>Sclerostin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>BSA</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

3.2.4 Assessment of the binding affinity

Table 3.2 shows the strategy to assess binding affinity of the chosen aptamer candidates to sclerostin. Eight specific aptamer candidates and one random sequence for control at different concentrations of 1 nM, 5 nM, 10 nM, 100 nM, and 500 nM were used to assess the binding affinity to sclerostin by ELONA. Aptamers would show a high absorbance at 450 nm if they have a high binding affinity to sclerostin. The detailed procedures to conduct the ELONA assay were described in chapter 2.
### Table 3.2  Strategy to assess binding affinity

<table>
<thead>
<tr>
<th>Group (n=3)</th>
<th>Concentration</th>
<th>Method</th>
<th>Evaluation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1-12: one aptamer in each group</td>
<td>1 nM, 5 nM, 10 nM, 100 nM, 500 nM</td>
<td>ELONA</td>
<td>Absorbance at 450 nm and $K_D$ value</td>
</tr>
<tr>
<td>Group 13: one random sequence</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### 3.3 Results and discussions

#### 3.3.1 Binding specificity of aptamer candidates against sclerostin

48 aptamer candidates were picked out from round 20 of SELEX according to their relatively high frequency of occurrence in the aptamer pool. These aptamer candidates together with one random sequence were evaluated by ELONA to determine their binding specificity. Specific aptamers showed relatively high absorbance at 450 nm when were bound to sclerostin, while quite low absorbance when bound to BSA. According to this principle, aptscl 6, aptscl 9, aptscl 15, aptscl 34, aptscl 46, aptscl 51, aptscl 56 and aptscl 132 were picked out for further use in the following binding affinity assessment.

Figure 3.1 visually shows the difference of binding specificity among different aptamer candidates against sclerostin and BSA. In the first diagram of figure 3.2, the absorbance value of aptamer candidates (aptscl 2, aptscl 6, aptscl 9, aptscl 15, aptscl 17, aptscl 27, aptscl 34, aptscl 36, aptscl 46 and aptscl 51) bound to sclerostin were beyond 2.5. However, the absorbance of aptscl 2 and aptscl 27...
bound to BSA were more than 0.125. So they were excluded in following assessment. Similarly, aptsc1 132 and 36 were picked out for binding affinity assessment. And aptsc1 140 was excluded. Though aptsc1 56 had a higher binding affinity to BSA than others’, its binding affinity to sclerostin was quite high. So it was also picked out for further assessment. Besides, we could see the binding affinities of random sequence to sclerostin and BSA were almost the same at a very low level.
Figure 3.1  Binding specificity of aptamer candidates against sclerostin.
3.3.2 Binding affinity of aptamer candidates against sclerostin

Binding affinity of twelve picked aptamer candidates against sclerostin (aptscl 6, aptscl 9, aptscl 15, aptscl 17, aptscl 27, aptscl 34, aptscl 36, aptscl 46, aptscl 51, aptscl 36, aptscl 56 and aptscl 132) were evaluated by ELONA. Different concentrations of aptamer candidates were added to the coated sclerostin. The results of binding affinity for eight aptamers (aptscl 6, aptscl 9, aptscl 15, aptscl 34, aptscl 46, aptscl 51, aptscl 56 and aptscl 132) could be well fitted into a curve. Figure 3.2 presents the binding affinity (K\textsubscript{D} value) of these aptamers against sclerostin. The K\textsubscript{D} values of these eight aptamers were all at level of nanomolar, indicating a high binding affinity to sclerostin for each aptamer. The detailed K\textsubscript{D} values of aptscl 6, aptscl 9, aptscl 15, aptscl 34, aptscl 46, aptscl 51, aptscl 56 and aptscl 132 were 4.2 ± 1.6 nM, 3.4 ± 2.3 nM, 11.0 ± 2.4 nM, 125.5 ± 53.4 nM, 45.6 ± 13.4 nM, 62.2 ± 20.0 nM, 43.1 ± 5.7 nM and 42.2 ± 1.7 nM respectively. Random sequence bound to sclerostin with a very low binding affinity, and with the amount of random sequence increasing, the binding affinity almost remained unchanged.
Figure 3.2 Binding affinity of aptamer candidates against sclerostin.
3.3.3 Predicted secondary structures of sequenced aptamers

Table 3.3 presents the sequence results of representative aptamer candidates. It is obvious that percentage of guanine were very high in these aptamers, with 50% of aptsc 6, 40% of aptsc 9, 42.5% of aptsc 15, 42.5% of aptsc 46, 47.5% of aptsc 51, 40% of aptsc 56 and 32.5% of aptsc 132. Due to the rich guanines, the sequences were subjected to analysis of QGRS mapper [69]. It is a software program that generates information on composition and distribution of putative Quadruplex forming G-Rich Sequences (QGRS) in nucleotide sequences. The possible G of involving in the formation of G-quadruplex were underlined in table 3.3 and the corresponding G-score were given. G-quadruplex structure is the most common structure selected in vitro SELEX, particularly for DNA aptamers. Aptamers with G-quadruplex structures have high diversity which enables them to bind with various targets.

Table 3.3 Sequence results of the representative aptamer candidates

<table>
<thead>
<tr>
<th>Aptamer</th>
<th>Sequence</th>
<th>G-score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aptsc 6</td>
<td>TGGAGAGGTGGTGGGCGGGGGGGTCCTCGCTCGAAGCTACG</td>
<td>40</td>
</tr>
<tr>
<td>Aptsc 9</td>
<td>TGGGCTAGGGGACCCTGCTAGTTAGTCAACGTTTTCGA</td>
<td>20</td>
</tr>
<tr>
<td>Aptsc 15</td>
<td>GAAGGGGCAACTATAGCTTATGTTGGGAGGGTGGACTACGTATC</td>
<td>7</td>
</tr>
<tr>
<td>Aptsc 46</td>
<td>GGGTGGATTTAAGGGGGCCCCTCGTGAAGGGCATTGCGAA</td>
<td>17</td>
</tr>
<tr>
<td>Aptsc 51</td>
<td>TGGGCTAGGGGTTCTGGCTAGTTGATTTGGCC</td>
<td>20</td>
</tr>
<tr>
<td>Aptsc 56</td>
<td>CGGGGGTGAGGGATTGCTCGTATTTTGCC</td>
<td>--</td>
</tr>
<tr>
<td>Aptsc 132</td>
<td>CCCAGACGAGACACCTCATGCTTTTCCTGGGGAGGGGT</td>
<td>20</td>
</tr>
</tbody>
</table>
The representative aptamers were further performed structure analysis by computational methods to gain more information about their structure. Mfold is a commonly used tool in molecular biology which is a web server for the prediction of DNA or RNA hairpin structures [70]. It could show the possible double strands and loops forming in aptamers. Generally, aptamers recognize and bind to their targets through their loop structures and the aptamer-target complexes are stabilized by double strand region. Figure 3.3 shows the possible secondary structure of the aptamer candidates with the lowest free energy.

![Secondary structures of aptamers predicted by mfold.](image)

Figure 3.3 Secondary structures of aptamers predicted by mfold.
3.4 Summary of chapter 3

In this chapter, binding specificity of 48 aptamer candidates were assessed by ELONA. Twelve aptamer candidates with high binding affinity to sclerostin while low binding affinity to BSA were chosen for the K_D assessment. The binding affinity assessment was also conducted by ELONA using aptamer candidates at different concentrations. The K_D values of these eight aptamer candidates were all at low levels of nanomolar. All together the aptamer candidates selected in the designed SELEX were successful to bind with sclerostin with high binding affinity and specificity.
Chapter 4

Evaluation of inhibition potency of selected ssDNA to sclerostin in cell level
4.1 Aims and overview

In this chapter, selected aptamers with high binding affinity and specificity were chosen for the evaluation of inhibition potency. They were assessed by means of measurement of sclerostin's antagonistic effect on Wnt signaling in osteoblast MC3T3-E1 cells.

4.2 Experimental section

4.2.1 Experimental materials

The chosen aptamers (aptscl 6 and aptscl 56) were synthesized and modified by 3’ inverted dT to increase their stability in serum.

MC3T3-E1 cells, vector ‘pSF-CMV-Puro-NH₂-GST-N-Terminal GST Tag mammalian plasmid’ and IPTG were purchased from Sigma Aldrich. Restriction enzyme EcoRI, restriction enzyme Xhol, T4 DNA ligase and ligation buffer were provided by New England Biolabs. Platinum® pfx DNA polymerase and Lipofectamine® 2000 transfection reagent were purchased from ThermoFisher. Plasmid mini-prep kit, midi-prep kit, PCR product purification kit, gel extraction kit (150) and gel purification kit (250) were all purchased from QIAGEN. Human sclerostin cDNA was form ACRO Biosystems. Human Wnt3a gene ORF cDNA clone expression plasmid was purchased from Sino Biological company. TOP Flash and FOP Flash luciferase plasmid were purchased from Addgene company.

DMEM was prepared and added with 10% FBS and 1% penicillin and streptomycin. 0.25, 2 and 3 µg/mL puromycin were added into the prepared
medium, respectively to determine the appropriate concentration of puromycin to select positively transfected cells.

4.2.2 Preparation of competent Cells

DH5α competent cells were chosen for plasmid culture.

4.2.2.1 Preparation of solutions

100 mM CaCl₂ solution and 100 mM CaCl₂-MgCl₂ solution were prepared according to the following procedures:

**100 mM CaCl₂:** 90 mL Milli-Q H₂O was added to 10 mL 1M CaCl₂•2H₂O stock solution. Then the obtained 100 mL solution solution was filtered with 0.45µm filter and laid on ice.

**100 mM CaCl₂-MgCl₂:** 90 mL Milli-Q H₂O was added to the mixed solution of 8 mL 1M MgCl₂ stock solution and 2 mL 1M CaCl₂•2H₂O stock solution. Then the 100 mL mixed solution was filtered with a 0.45µm filter and laid on the ice.

4.2.2.2 Protocol for competent cell preparation

Single colonies of *E. coli* were prepared with streak method on LB agar plate and incubated for overnight at 37 °C. A single colony was picked and inoculate in 2 mL LB broth for overnight, followed by incubation in 100 mL LB broth (the inoculation ratio is 1:50) for 1 to 3 hours until OD (at 600 nm) value reaching to 0.4. The cultured *E. coli* was then transferred into a clean and sterile 50 mL falcon tube and cooled down to 0 °C in ice for 10 min. The cells were
harvested by centrifugation at 2700 g at 4 °C for 10 min. Then LB broth was removed by decanting and standing 50mL falcon upside down on clean paper tissue for 1 minute. The cell pellet was gently and carefully resuspended and washed for twice with 30 mL 100 mM MCl₂-CaCl₂ solution. The MgCl₂-CaCl₂ solution was pre-cooled on ice. The cells were harvested by centrifugation at 2700 g at 4 °C for 10 min following each wash. Then supernatant was removed by decanting and standing 50 mL falcon upside down on clean paper tissue for 1 minute. The cell pellet was resuspended gently and carefully with 3 mL 0.1 M CaCl₂ which was also pre-cooled on ice. 1mL autoclaved glycerol was added into the cell suspension. The whole solution was mixed gently using a pipette. The role of glycerol was to protect the cells from being damaged during the process of freezing and thawing. At last, 100 µL cell suspension was aliquoted into a 1.5 mL autoclaved tube and was stored at -80°C for use.

4.2.3 Construction of plasmid containing sclerostin

Figure 4.1 shows the strategy to clone human sclerostin cDNA into the expression vector pSF-CMV-Puro-NH₂-GST. First, human sclerostin cDNA was amplified by PCR. The parameters in PCR were set as follows: Initial denaturation condition was 95 °C lasting for 2 min. 94 °C was kept for 15 seconds for denaturation. The denaturation round was repeated for 30 cycles. Then 55 °C was kept for 30 seconds for annealing. 68 °C was kept for 75 seconds for elongation and 68 °C was kept for 10 min for final extension. 1 ng template, 0.3
µM forward primer and reverse primer, 0.3 mM dNTPs, pfx buffer, 1.25 units of pfx DNA polymerase and 1 mM MgSO$_4$ were included in the PCR reaction with a final volume of 50 µl.

When the PCR process was completed, 5 µL PCR products were applied to 1% agarose gel electrophoresis to check whether the gene was successfully amplified. Then remaining 45 µL PCR products were purified with gel purification kit. Then the purified PCR products were digested using corresponding restriction enzymes (restriction enzyme EcoRI and restriction enzyme Xhol). The vectors were also digested with this two kinds of restriction enzymes or cloning. Next, the digested PCR products were applied s onto the 1% agarose gel electrophoresis. Bands with right size of human sclerostin cDNA and vector were cut from the gel and purified using plasmid mini-prep kit. Then they were ligated together by cohesive ends using T4 DNA ligase at 16 °C and was incubated for overnight.

The ligated combination of vector and sclerostin cDNA was transformed into DH5α competent cells by heat shock at 42 °C for 90 seconds. Then, 1 mL LB broth was added into competent cells (total volume was 1.1 mL) and they were incubated for 1 hour at 37 °C in a shaker. The incubated competent cells were spin down and 1 mL of the supernatant was removed. The remaining 100 µL competent cells were resuspended and spread to the LB agar with kanamycin. They were incubated at 37 °C for overnight. After overnight incubation, bacteria
colonies were picked up and each colonies were cultivated with 5 mL LB broth at 37 °C for overnight. Then extraction and purification the plasmids for the bacteria were performed using plasmid midi-prep kit. The concentration of purified plasmids was determined using Nanodrop (OD ratio 260/280). To check whether the target gene from sclerostin cDNA was inserted into the plasmid, the plasmids were used as templates in PCR. The PCR products were analyzed by 1% agarose gel electrophoresis. If target gene was successfully amplified, then it indicated that the target gene was inserted into the plasmids. At last, the purified sclerostin plasmid was sequenced.

Figure 4.1 Target gene was inserted into the expression vector pSF-CMV-Puro-NH2-GST.

pSF-CMV-Puro-NH2-GST.
4.2.3 Cell culture and transfection of plasmid

MC3T3-E1 cells were cultured in DMEM media with 10% FBS in advance for use. If transfection of sclerostin plasmid, Wnt3a plasmid, FOP luciferase plasmid or TOP luciferase plasmid into the MC3T3-E1 cells was needed, lipofectamine® 2000 transfection reagent would be used (per transfection reagent with 3 equivalent of plasmid). The stable transfected cells were added puromycin (at a final concentration of 2 µg/mL) and cultured more than 2 weeks.

4.2.4 Assessment of inhibition potency of aptamers to sclerostin

To assess the inhibition potency of aptamer candidates to sclerostin’s antagonistic effect on Wnt signaling, a TOP-Wnt induced luciferase reporter assay were used. Osteoblast MC3T3-E1 cells were chosen in this study because they are not capable of expression of sclerostin but can express receptors of sclerostin as well as proteins in downstream of Wnt signaling pathway [46]. The Wnt pathway can only become active in the presence of Wnt3a. When sclerostin is also expressed, it will antagonize Wnt3a to bind with the receptors. Therefore, the Wnt signaling pathway will be inhibited. However, when aptamers against sclerostin are added, they will interact with sclerostin and inhibit the antagonistic effect of sclerostin to Wnt signaling. So the Wnt signaling will be reactivated. The underlying principle of the inhibition potency assessment assay is also presented in Figure 4.2.
Figure 4.2  Scheme of assessment of inhibition potency to sclerostin’s antagonistic effect on Wnt signaling.

MC3T3-E1 cells were seeded in 24-well plates and eighteen wells of cells were cultured. These wells were divided into six groups with each group containing three wells for parallel experiment. Table 4.1 shows the detailed strategy to assess the inhibition potency of aptamers to Inhibition potency of aptamers to sclerostin’s antagonistic effect on Wnt signaling.

Cells in group 1 were co-transfected with FOP luciferase plasmid and Wnt3a plasmid as a negative control. Cells in group 2 were co-transfected with TOP luciferase plasmid and Wnt3a plasmid. Cells in group 3, group 4, group 5 and group 6 were all transfected with TOP luciferase plasmid, Wnt3a plasmid and sclerostin plasmid. After 6 hours of transfection, the culture medium of cells in
these six groups were all changed to fresh medium. Cells in group 1, group 2 and group 3 remained untreated. Cells in group 4 were added into random sequence. Cells in group 5 were added into one aptamer candidate selected from aptamers (modified with 3′-inverted thymidine) having high binding specificity and affinity. Cells in group 6 were added into sclerostin antibody (AMG-785) to make a comparison between sclerostin aptamer and sclerostin antibody in inhibition potency. The added ssDNA or antibody and cells were incubated for 24 hours.

Then the cells were lysed with 100 µL lysis buffer and 20 µL cell lysate were used for analysis. Luciferase assays were performed using Dual-Luciferase Reporter system with parameters set according to the manufacture’s protocol. The corresponding luciferase signals were measured.

**Table 4.1 Strategy to assess inhibition potency to sclerostin’s antagonistic effect on Wnt singling**

<table>
<thead>
<tr>
<th>Group ( n=6 )</th>
<th>Treat</th>
<th>Method</th>
<th>Evaluation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1: co-transfected with FOP luciferase plasmid and Wnt3a plasmid</td>
<td>Untreated</td>
<td>Luciferase activity assay</td>
<td>Luciferase signal</td>
</tr>
<tr>
<td>Group 2: co-transfected with TOP luciferase plasmid and Wnt3a plasmid</td>
<td>Random sequence</td>
<td>Lysis buffer and 20 µL cell lysate</td>
<td></td>
</tr>
<tr>
<td>Group 3: co-transfected with TOP luciferase plasmid, Wnt3a plasmid and sclerostin plasmid</td>
<td>Random sequence</td>
<td>Lysis buffer and 20 µL cell lysate</td>
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<tr>
<td>Group 4: co-transfected with TOP luciferase plasmid, Wnt3a plasmid and sclerostin plasmid</td>
<td>Random sequence</td>
<td>Lysis buffer and 20 µL cell lysate</td>
<td></td>
</tr>
<tr>
<td>Group 5: co-transfected with TOP luciferase plasmid, Wnt3a plasmid and sclerostin plasmid</td>
<td>Random sequence</td>
<td>Lysis buffer and 20 µL cell lysate</td>
<td></td>
</tr>
<tr>
<td>Group 6: co-transfected with TOP luciferase plasmid, Wnt3a plasmid and sclerostin plasmid</td>
<td>Antibody</td>
<td>Lysis buffer and 20 µL cell lysate</td>
<td></td>
</tr>
</tbody>
</table>
4.3 Results and discussions

Two of the aptamer candidates (aptscl 6 and aptscl 56) with both high binding specificity and affinity were picked out. The inhibition potency of them to sclerostin’s antagonistic effect on Wnt signaling were assessed in osteoblast MC3T3-E1 cells. They were modified with 3’ inverted thymidine to increase their stability in culture medium with serum [58,71]. According to the underlying principle of inhibition potency assessment, a high reactivated response means a high inhibition potency of aptamers to sclerostin’s antagonistic effect on Wnt signaling.

Figure 4.3 shows the inhibition potency of aptscl 6 and sclerostin antibody to sclerostin’s antagonistic effect on Wnt signaling. It could be seen that aptscl 6 with concentration of no less than 23.7 µg/mL exerted the similar effect with sclerostin antibody with concentration of no less than 10 µg/mL to reactivate the Wnt signaling. The responses of aptscl 6 at concentration of 47.4 µg/mL and 59.25 µg/mL were almost the same, probably reaching a saturated status. Responses of aptscl 6 at concentration of 5.925 µg/mL and 11.85 µg/mL were close to that in the blank experiment without aptamers or antibody. Similarly, responses of antibody at the concentration of 2.5 mg/mL and 5 mg/mL were relatively as low as that in blank experiment.
Figure 4.3  Inhibition potency of aptsc1 6 and antibody to sclerostin’s antagonistic effect on Wnt signaling in MC3T3-E1 cells.

Figure 4.4 shows the inhibition potency of aptsc1 56 and sclerostin antibody to sclerostin’s antagonistic effect on Wnt signaling. Similar results with aptsc1 6 could be seen in aptsc1 56. But it seemed that the response of aptsc1 56 at saturation status (15000) was higher than that of aptsc1 6 (13000). And the concentration of aptsc1 56 at saturation status (25 µg/mL) was lower than that of aptsc1 6 (47.4 µg/mL). However, the binding affinity of aptsc1 56 to sclerostin (K_D=43.1 ± 5.7 nM) was no stronger than that of aptsc1 6 (K_D=4.2 ± 1.6 nM). So it seemed that a high binding affinity might not associated with a strong inhibition potency.
Figure 4.4  Inhibition potency of aptsc1 56 and antibody to sclerostin’s antagonistic effect on Wnt signaling in MC3T3-E1 cells.

To further evaluate the inhibition potency of aptsc1 6 and aptsc1 56, the results were fitted into two curves respectively. Figure 4.5 presents the EC_{50} values of aptsc1 6 and aptsc1 56 to reactivate Wnt signaling respectively. Consistent with the above preliminary deduction, inhibition potency of aptsc1 56 with a EC_{50} of 1.55 µM was a lit bit better than that of aptsc1 6 with a EC_{50} of 1.6 µM.

Figure 4.5  EC_{50} values of aptsc1 6 and aptsc1 56 to reactivate Wnt signaling.
4.4 Summary of Chapter 4

In this chapter, aptsc1 6 and aptsc1 56 were selected from last chapter and synthesized with modification of 3’ inverted T. They were chosen to assess the inhibition potency to sclerostin by measuring the signal of luciferase response in MC3T3-E1 cells. Through experiment results, we can know that 47.4 µg/mL aptsc1 6 or 25 µg/mL aptsc1 56 could behave as well as 20 mg/mL sclerostin antibody in terms of inhibiting sclerostin in MC3T3-E1 cells. Both of aptsc1 6 and aptsc1 56 have a good inhibition potency with a EC\textsubscript{50} value of 1.6 µM and 1.55 µM.

All together, aptamer candidates selected in the designed SELEX were successful to bind to sclerostin with high binding affinity and specificity \textit{in vitro}, as well as successful to reactivate Wnt signaling with good potency. These results imply that sclerostin aptamers have the great potential to be novel agents to promote bone formation and therefore to treat with osteoporosis.
Chapter 5

Conclusions and prospective
In this study, 20 rounds of SELEX were performed to select the aptamers that have high affinity and specificity to sclerostin. The results showed a successful enrichment of aptamers with high binding affinity in aptamer pool of round 20 indicating the SELEX built in this study was feasible. Forty-eight aptamers were picked out for the binding specificity assessment which was performed by ELONA using BSA as the contrast protein. Twelve aptamer candidates with high binding affinity to sclerostin while low affinity to BSA were chosen for further assessment. The $K_D$ values of these eight aptamers in binding affinity assessment assay were all at low levels of nanomolar, indicating high binding affinities of these aptamers to sclerostin.

The inhibition potency of aptamers to the antagonistic effect of sclerostin on Wnt signaling in osteoblast MC3T3-E1 were also evaluated. Two of the aptamer candidates (aptscl 6 with $K_D=4.2 \pm 1.6$ nM and aptscl 56 with $K_D=43.1 \pm 5.7$ nM) were chosen to perform the TOP-Wnt induced luciferase reporter assay to determine the inhibition potency. The results demonstrated that sclerostin aptamers could exert similar effect to reactivate Wnt signaling with anti-sclerostin antibody. Besides, $EC_{50}$ values of the two aptamer candidates were also at low levels of micromolar (1.55 $\mu$M for aptscl 56 and 1.6 $\mu$M for aptscl 56). From the comparison of inhibition curves of aptscl 56 and aptscl 6, a high binding affinity (low KD value) did not necessarily mean a strong inhibition potency (low $EC_{50}$).
value). These results in inhibition potency assessment assay indicate that sclerostin aptamers have a great potential to promote bone formation.

The research establishes a foundation for the next stage preclinical studies and it will benefit the development of novel bone anabolic agents to reverse osteoporosis. For the following works, chemical modifications of the selected aptamers (aptscl 6 and aptscl 56) should be performed to increase the stability of them in serum and in vivo. Afterwards, to further verify the effect of selected aptamers in vivo, corresponding animal models should be built. Only through thorough experiment designs, could the nucleic acid aptamers be the promising alternative agents to antibodies in the treatment of osteoporosis.


4. [https://www.niams.nih.gov/Health_Info/Bone/](https://www.niams.nih.gov/Health_Info/Bone/)


41. Li, X.; Ominsky, M. S.; Niu, Q.-T.; Sun, N.; Daugherty, B.; D’Agostin, D.; Kurahara, C.; Gao, Y.; Cao, J.; Gong, J.; Asuncion, F.; Barrero, M.; Warmington,


CURRICULUM VITAE

Academic qualifications of the thesis author, Ms. LYU Quanxia:

- Received the degree of Bachelor of Environmental Sciences from University of Jinan, July 2011.
- Received the degree of Master of Environmental Sciences from Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, July 2015.

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