Enhancement of Osteogenesis and Mineralization by Delivery of Noggin siRNA from Lipid-Based Nanoparticles

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Abstract

Several types of serious bone defects would not heal without invasive clinical intervention. One approach to such defects is to enhance the capacity of osteoblasts. Exogenous bone morphogenetic proteins (BMP) have been utilized to positively regulate matrix mineralization and osteoblastogenesis, however, numerous adverse effects are associated with this approach. Noggin, a potent antagonist of BMPs, is an ideal candidate to inhibit and decrease the need for supra-physiological doses of BMPs. In this research, a novel siRNA-mediated gene knock-down strategy to down-regulate Noggin is reported. We utilized a lipid nanoparticle (LNP) delivery strategy in pre-osteoblastic rat cells. *In vitro* LNP-siRNA treatment caused inconsequential cell toxicity and transfection was achieved in over 85% of cells. Noggin siRNA treatment successfully down-regulated cellular Noggin protein levels and enhanced BMP signal activity, which in turn, resulted in significantly increased osteoblast differentiation and extracellular matrix mineralization, evidenced by histological assessments. Gene expression analysis indicated that targeting Noggin in bone cells would not lead to a compensatory effect from other BMP negative regulators such as *Gremlin* and *Chordin*. The results from this study support the notion that novel therapeutics targeting Noggin have the translational potential to enhance bone formation without the need for toxic doses of exogenous BMPs. Such treatments will undeniably provide safe and economical treatments for individuals whose poor bone repair results in permanent morbidity and disability.

Résumé

Plusieurs types de défauts osseux graves ne guérissent pas sans intervention clinique invasive. Une approche de tels défauts est d'améliorer la capacité des cellules de formation osseuse. Des protéines morphogénétiques osseuses exogènes (BMP) ont été utilisées pour réguler positivement la minéralisation de la matrice et l'ostéoblastogenèse, mais de nombreux effets indésirables sont associés à cette approche. Noggin, un antagoniste puissant des BMPs, est un candidat idéal pour cibler et diminuer le besoin de doses supraphysiological de BMPs. Dans la présente recherche, une nouvelle stratégie de knock-down de gène médiée par siRNA pour réguler à la baisse Noggin est reporté. Nous avons utilisé une stratégie d'administration de nanoparticules lipidiques (LNP) dans des cellules de rats pré-ostéoblastiques. Un traitement *in vitro* par LNP-siRNA a provoqué une toxicité cellulaire sans conséquence et la transfection a été obtenue dans plus de 85% des cellules. Le traitement par siRNA de Noggin a abaissé avec succès les taux de protéine Noggin cellulaire et augmenté l'activité du signal BMP, ce qui a entraîné une différenciation ostéoblastique significativement accrue et une minéralisation de la matrice extracellulaire mise en évidence par des évaluations histologiques. L'analyse de l'expression génique a montré que cibler Noggin dans les cellules osseuses ne conduirait pas à un effet compensatoire d'autres régulateurs négatifs BMP tels que *Gremlin* et *Chordin*. Les résultats de cette étude confirment l'idée que de nouveaux agents thérapeutiques ciblant Noggin ont le potentiel cliniquement pertinent d'améliorer la formation osseuse sans avoir besoin de doses toxiques de PGB exogènes. De tels traitements fourniront

indéniablement des traitements sûrs et économiques pour les personnes dont la réparation osseuse médiocre entraîne une morbidité et une incapacité permanentes.

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Firstly, I wish to express my sincerest gratitude to my supervisors, Dr. Hamdy and Prof. Tabrizian for their continuous guidance, support, patience and knowledge. I experienced a unique collaborative environment to link two seemingly distant areas of knowledge with each other: orthopaedic surgery and bioengineering. I was offered tremendous freedom to work with multidisciplinary groups of experts to learn from them, and I thank you for providing me with these opportunities.

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My sincere thanks go to Ms. Paula Wall and Timothy Johns, our lab manager and all the previous and current lab members working at Biomatx under Dr. Tabrizian's supervision and at Shriners Hospital under Dr. Hamdy's supervision: Amir, Mina, Laila, Samira, Lamees, Kaushar, Feriel, Pareesa, Saadia, Selya, Khalil, Nick, Timuthee, Meltem, Rafael, Sandrine and Sultan. You made all days and nights of work in the lab feel easy, fun, joyful and memorable. I appreciate all your help, brainstorming and discussions which made every step of my PhD experience at McGill a special one. Thanks to all my friends within orthopaedic residency program and Experimental Surgery Student Association for your support and trust in my leadership and the opportunity to serve you as Vice President of Academic Affairs; it was such an important and crucial role with countless learning opportunities.

I appreciate and acknowledge all the technical and specialized help I received from the staff at Shriners Hospital animal facility (Mia), FACS facility at McGill (Julien Leconte), SAIL RI-MUHC (Mathieu Simard) and IRIC Genomic Center at UdeM (Raphaelle Lambert and Valérie Villeneuve) and Dr. Pierre Moffatt, Marie Helene and Dr. Rene St-Arnaud for their help with animal cell line and their scientific input.

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Preface and Author Contributions

This dissertation is a collection of published and un-submitted work in which the author is the main contributor in experimental design, performing the experiments, data collection, data analysis and interpretation, as well as drafting, writing the manuscripts, data presentation and editing the texts. All the original contributions in the form of publications could be found in the supplementary section of the current thesis. The review article published in the Journal of Bone and Mineral Research is an original contribution and several parts of this publication have been utilized in the formation of a background basis for this thesis. Dr. Mina Mekhail and Dr. Ahmed Aoude's names appears in this publication as second and third co-authors whom contributed in the global design and editing the final manuscript. Lastly, Dr. Maryam Tabrizian's and Dr. R. Hamdy's names appear in all works for their leadership and supervisory roles and guidance.

Outline of the thesis

The purpose of this dissertation is to present and elaborate on the work achieved during my PhD training to test and introduce an alternative methodology for the treatment of bone defects and to accelerate bone regeneration. I worked on a new perspective of the topic of bone healing which has been studied extensively previously. This was mainly done by re-contextualizing the current treatment approaches to accelerate bone formation (i.e. exogenous bone specific growth factors such as BMP-2) and the addition of a genetic tool to inhibit the negative regulators of BMPs. I have shown that the above approach is applicable to osteoblasts treated by exogenous recombinant BMP-2. This combination therapy had not been tested previously in the setting utilized by my research. I showed that the concept of such a combination therapy is feasible and useful.

This thesis has been designed and presented as a monograph. As such, it starts by a deep and broad coverage of the underlying challenges of impaired and slow bone regeneration and current approaches to address this issue. A deep review of the literature is presented in a way to cover both the clinical difficulties resulting from non-healing or slow-healing bone defects and the challenges in utilizing the current treatment modalities in the clinical setting. Within the introduction, the alternative approaches which could be solely at the pre-clinical stage but with a promising future to be translated into the bedside were reviewed. The next chapter provides details of the materials and methods used in this study. The methods are presented chronologically as carried out during the study, for both experiments which led to publishable data and

for the primary experiments, as well as the proof of concept trials. Next chapter presents the results achieved, followed by another chapter entailing a global discussion and future directions for the current project. The cumulative bibliography of all the citations for this dissertation comes next. Finally, the supplementary section of the thesis contains copies of the publications, all the permissions acquired from the publishers of the works from which one or more parts have been used in the current thesis and a curriculum vitae of the author with a detailed list of previous publications and achievements.

Problem Statement, Hypothesis and Rationale

Problem statement

Despite bone's natural regeneration ability following an injury, several orthopaedic conditions cause non-unions or delayed union. Additionally, critical size defects (CSD), a type of serious bone defect that would not heal without invasive clinical intervention are a huge challenge in orthopedic surgery.

CSD could occur following trauma (150,000 cases occur annually in the US), tumor excision, developmental anomalies or infections. Currently, the most widely used surgical technique to treat CSD, is a form of bone grafting called autologous. In this procedure, the bone defect is filled with bone that has been removed from another part of the same patient's body, through an additional operation. Due to high morbidities associated with this technique, there is a huge interest in ways to augment the naturally slow growth of bone to achieve fast and effective bone healing.

Bone morphogenetic proteins (BMPs) are growth factors that stimulate bone formation. Different BMPs have lately been identified. Even two types of BMP (BMP-2 and BMP-7) have been synthesised in the lab and are commercially available and have even been used clinically. However, in this approach, doses of BMP thousands of times higher than what naturally occurs in body would be necessary to achieve a therapeutic effect. Aside from the enormous costs, such doses cause serious safety issues, such as toxicity, ejaculation problems and tumors. The need for huge doses of BMP is mainly due to a cellular self-limiting negative feedback mechanism by BMP antagonists. One of the key antagonists of BMPs is a protein called Noggin. **Figure *1***

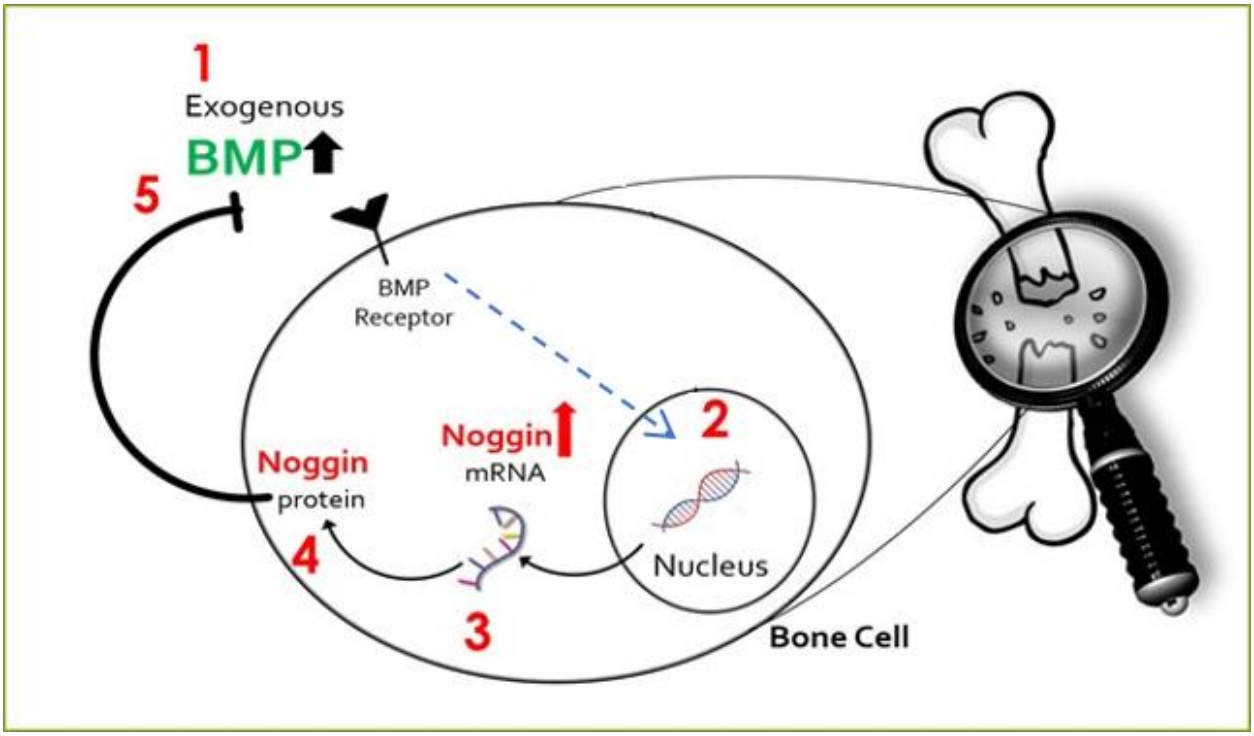


Figure 1. *Schematic mechanism of action of BMP and the initiation of Noggin negative feedback cascade in osteoblasts.*

Our aim was to stimulate bone formation using just safe and low concentrations of BMP. Our proposed approach was to inhibit Noggin to increase the efficacy of low-dose BMP in the absence of its antagonist. **Figure *2***.

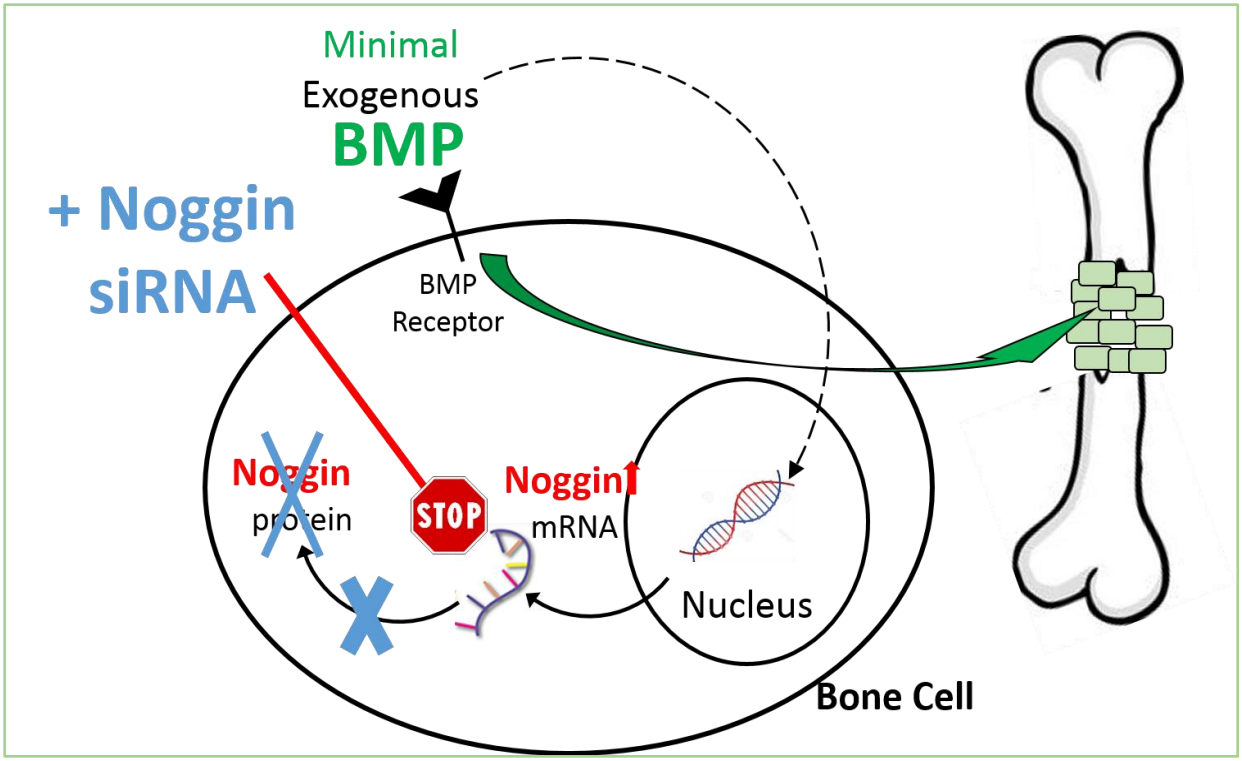


Figure 2. *Schematic mechanism of action of BMP and the initiation of Noggin negative feedback cascade which is blocked by means of siRNA against Noggin.*

A novel approach to decrease the expression of a protein such as Noggin is to interfere with its cellular production by small interfering RNA (siRNA). siRNA is a small sequence of nucleotides (units building DNA or RNA) and a novel genetic tool that could be easily designed and produced against any protein-encoding gene.

Inhibition using siRNA is a practical approach to specifically target Noggin and decrease its mitigating effects on BMP. It enhances bone formation and the differentiation of stem cells to bone cells (osteogenesis). A key limitation to successful application of siRNA as a therapeutic strategy is its delivery to the site of action. Various

non-viral siRNA delivery vehicles have been developed, among them, lipid nanoparticles (LNPs) are in the most advanced stage of development. In the current study we aimed to investigate the bone regenerative effects of Noggin siRNA encapsulated in LNPs on pre-osteoblasts.

Hypothesis

The delivery of Noggin siRNA from LNPs would be efficient and can lead to enhanced osteoblastic differentiation and bone formation compared to control siRNA.

Rationale

Despite the revolutionary role of BMPs in accelerating bone formation in several orthopedic procedures, there has been growing evidence attributing various adverse events to the use of supra-physiologic doses of exogenous BMP in clinic. This highlights the need for an alternative approach towards promoting physiologic bone repair. Noggin is a known target to inhibit and achieve increased BMP efficacy as it is a key extracellular BMP antagonist and its inhibitory role in BMP-mediated osteogenesis has been extensively investigated both in animal models and rodent cells. On the other hand, several studies have exhibited the effectiveness of blockage of Noggin and consequent increase in BMP levels.

LNPs are presently the principal delivery systems that utilize the therapeutic potential of siRNA in cells. Successful and efficient delivery of siRNA has been reported utilizing LNPs; additionally, LNP-siRNA is able to achieve gene silencing at low doses. There are various reports from clinical trials examining LNP-siRNA systems

demonstrating positive results and clinical benefits, however, none exists in the field of orthopedic surgery, so far. Quite a few LNP-siRNA formulations are presently in different stages of clinical development by leading siRNA therapeutic companies; all of them have demonstrated safe clinical profiles and promising activity. Nevertheless, LNP approaches for local delivery of Noggin siRNA to bone has not been investigated previously and the efficiency of this approach is unknown. As such, we planned to test our hypothesis by investigating the effects of Noggin siRNA encapsulated in LNPs on osteogenesis and mineralized matrix formation.

Approach

To address our hypothesis, the following aims were set to be achieved:

Aim 1) Layer by layer lipid core nanoparticle (L-B-L LNP) for siRNA delivery:

- Synthesizing, characterizing and evaluating the *in vitro* efficiency of L-B-L LNPs

Aim 2) Evaluate the effects of recombinant BMP-2 on *Noggin* expression *in vitro*

Aim 3) Perform *in vitro* tests to examine the bioactivity and cytotoxicity of LNPs encapsulating siRNA

Aim 4) Perform functional tests to evaluate the effects of the LNPs encapsulating the Noggin siRNA on osteoblast differentiation and mineralization

Aim 5) Evaluate the effects of Noggin inhibition on other negative regulators of BMP signal

Proof of concept experiments:

A) Test the LNP-Noggin siRNA system on healthy Human osteoblast/ bone marrow samples to develop the methodology for human cells

B) Evaluate the properties of a 3D collagen scaffold in encapsulating LNP-siRNA

C) Perform *in vivo* experiments to evaluate the LNP-siRNA system for bone regeneration

The results of this study shed light on the application of one of the latest gene delivery approaches (i.e. siRNA therapeutics) in the field of bone regeneration and orthopaedic surgery. According to the best of our knowledge, there is no similar work utilizing our methodology and investigating all the aspects which have been studied by our group, and therefore this original research fills an essential gap in the literature. The results of this work will provide a novel approach to achieving accelerated bone repair by increasing the efficacy of conventional treatments.

siRNA technologies have shown significant promises in various diseases, and just recently their promising use in orthopedic conditions has been slowly unfolding. The enormous potential siRNA therapeutics hold is mainly due to the discovery of various signaling pathways responsible for different skeletal complications; different siRNA approaches enable investigators to target and modulate defective signaling pathways specifically at desired levels.

1. Introduction

1.1. Bone

Bone makes the largest part of body's connective tissue. Bone matrix is constantly mineralized physiologically and undergoes regeneration during the life. This organ is made up of different types of cartilage (cartilaginous joints, growth plate during youth), bone marrow and cortical and cancellous structures. As a tissue, bone has mineralized and nonmineralized (osteoid) regions (M. Lyons 2013).

Three different cell types are found in bone: bone forming cells called osteoblasts, osteocytes (when osteoblasts are surrounded completely by mineralized tissue), and lastly, bone resorbing cells, osteoclasts. These three cell types are in constant contact with each other and communicate either directly or via cell signalling mechanisms.

The extracellular matrix (ECM) of bone comprises much of this tissue and is produced mainly by osteoblasts; it also contains some proteins from blood. The ECM consists of the mineral part, different types of collagen, water, proteins other than collagens and a small proportion of lipids; in human, this proportion differs by age and site of the body (Shekaran and Garcia 2011). Each of these components possesses distinctive metabolic and mechanical functions. The studies on mouse models, tissues from healthy or diseased human or *in vitro* studies have all contributed to a better understanding of the physiological functions of bone components (Boskey, Doty et al. 2008).

The mineral part of bone provides strength and mechanical resistance to the collagen structure and is also a great source of calcium, phosphate and magnesium for

the body. The amount and size of mineral crystals play important roles in the disorders of bone. In osteoporosis, and as the result of impaired remodeling process, the small crystals are lost and the larger ones remain intact, this leads to the brittle osteoporotic bones. Whereas in osteopetrosis, the mineral crystals of bones are small compared to the healthy bones (Pagani, Francucci et al. 2005).

The matrix network of bone is mainly composed of type 1 collagen; however, other types of collagen might be found during the development of the bone.

Non-collagen proteins that are found in the extracellular matrix, form 10-15% of total proteins of this tissue. Albumin, glycoprotein, alkaline phosphatase and osteonectine are examples of such proteins. They have key roles in cell surface signalling, matrix organisation, bone cell proliferation and differentiation. Mutations in the genes coding these proteins could lead to serious diseases of skeletal system (Verrecchia, Rossert et al. 2001, Delany and Hankenson 2009).

1.2. Bone repair

Unlike many organs in the body, bone is capable of healing and restoring its original shape and function spontaneously, following an injury (Schmidt-Bleek, Marcucio et al. 2016). Most fractured bones heal by 20 weeks (Littenberg, Weinstein et al. 1998) depending on the location and severity of fracture as well as the presence of infection or open wound at the site of fracture. Nevertheless, this repair process does not always

occur satisfactory and could lead to mal-union or even non-union of the injured bone.(Bhandari, Adili et al. 2001) In the US alone, non-unions following fracture are seen in almost 10% of the cases (Rommens, Coosemans et al. 1989, Fernandez, Ring et al. 2001). This accounts for a huge economic burden on healthcare systems and the patients.

Bone repair occurs via an orchestrated and sophisticated series of biological events. Normal bone healing following a fracture of long bones could occur through two major pathways: Primary bone healing or Secondary bone healing **Figure *3***.

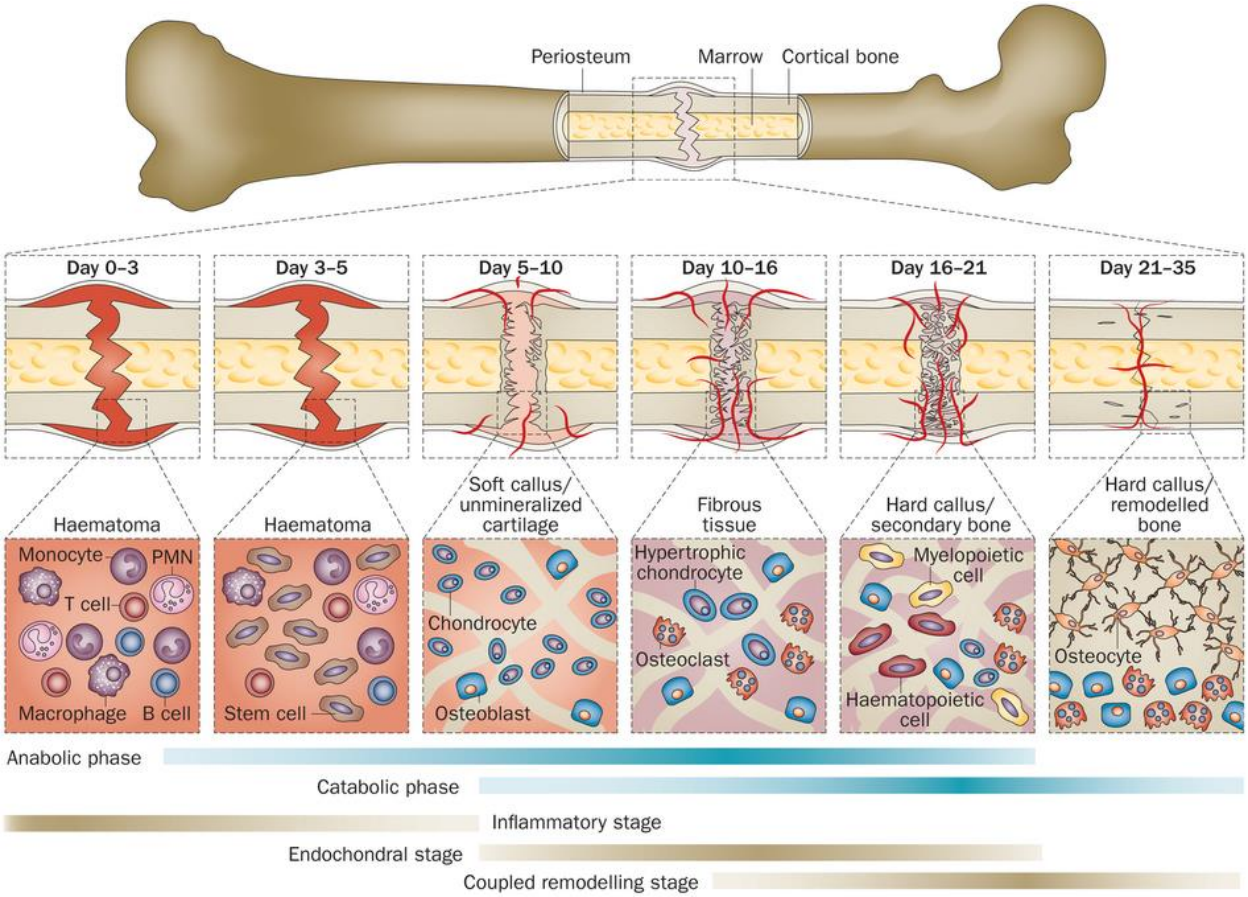


Figure 3. *Divergent phases of fracture healing. The metabolic events (Blue) overlap with biological phases (Brown). Three stages of metabolic phase are inflammatory, endochondrial bone formation and remodelling. The duration of healing is based on a femur fracture in mice with an intramedullary fixation. (Einhorn and Gerstenfeld 2014) with permission*

1.2.1. Primary bone healing

Primary bone healing is rare compared to the secondary bone healing (Einhorn 1998). This type of healing occurs when the two ends of fractured bone are fixed completely without moving (Delimar, Smoljanovic et al. 2012). In this type of healing a

callus is not usually shaped and the bone heals through intramembranous bone formation. Osteoblasts which are responsible for producing mineralized matrix, are recruited directly from surrounding periosteum to fill the gap of fractured bone **Figure 4**. A woven bone is laid down without any interphase of cartilage or fibrous tissue (Kriss, Taren et al. 1969). Through a remodelling process, Haversian canals are shaped by removing the woven bone and replacing it by newly formed bone.

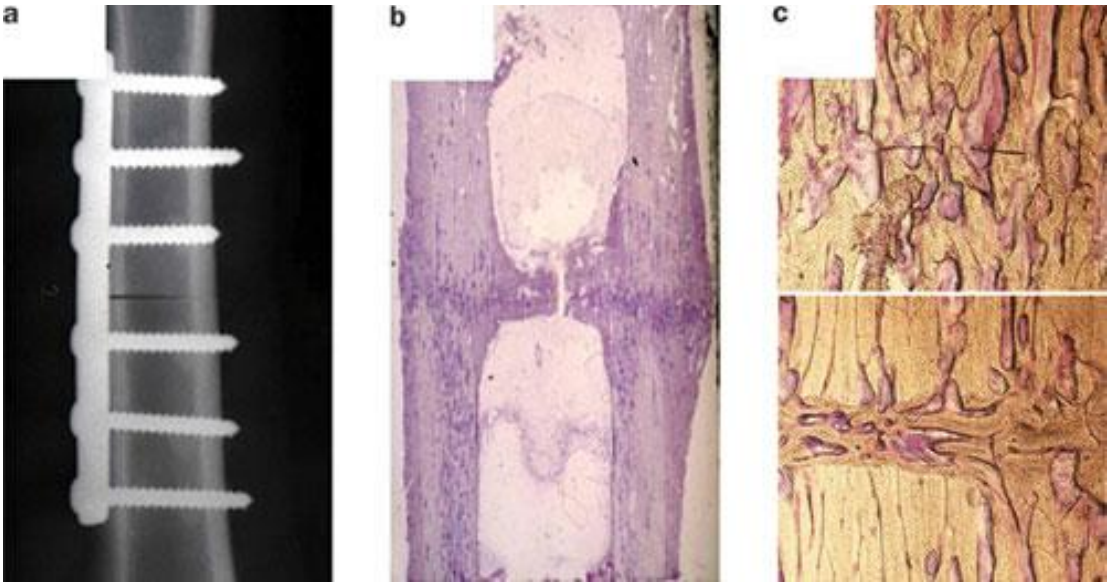


Figure 4. Primary bone formation. This type of healing occurs when a tight fixation of the fractured bone is provided. The healing through Haversian remodeling occurs immediately. Adapted from (Claes, Recknagel et al. 2012) with permission.

1.2.2. Secondary bone healing

Secondary bone healing is what usually occurs naturally following a fracture in the absence of a rigid fixation at the site of fracture. There are several overlapping phases through which the secondary healing occurs. The first phase is the inflammatory

phase which usually lasts for one week **Figure *5***. Immediately following fracture, there is substantial amount of bleeding and a hematoma is shaped around the site of fracture. Blood brings several cytokines, growth factors, cells and inflammatory agents to the site. The inflammatory tissue also provides an initial early mobilization for the fractured bones.

The next phase is the reparative phase which starts only a few days following the fracture, as such, it overlaps with the first phase and continues for a few weeks (Fernandez-Tresguerres-Hernandez-Gil, Alobera-Gracia et al. 2006, Troedhan, Kurrek et al. 2012). During this phase, a cartilaginous template of the bone to be made is made that bridges the two broken ends of fractured bone. This cartilaginous template is then replaced by the mineralized matrix which is produced by osteoblasts and a bony callus is formed at the fracture gap through endochondral bone formation. The differentiation of precursor cells to osteoblasts in this phase could be compromised if the conditions (vascular formation and blood supply, oxygenation, growth factors, etc.) are not optimal; instead, these cells could differentiate to fibroblasts (producing a fibrous tissue instead of bone) or chondroblasts (Producing cartilage) resulting in lack of bone formation. This phase of the fracture repair could take between four to 16 weeks, depending on the location of the fracture and comorbidities (infection, etc.) and several individual conditions (genetics, health and diet, etc.).

Over a much longer process (several months to four years), during the next phase which is called remodelling, excess areas of the bony callus are absorbed by osteoclasts (specialized bone resorbing cells) and finally the original shape and function of the bone is achieved. Osteoclasts and osteoblasts work simultaneously at this stage

to bring order to the disorganized woven bone and until the bone regains its original shape and functionality. Of course, this fine-tuned process could potentially be disturbed at any step resulting in mal-union or non-union (Van Nielen, Smith et al. 2017).

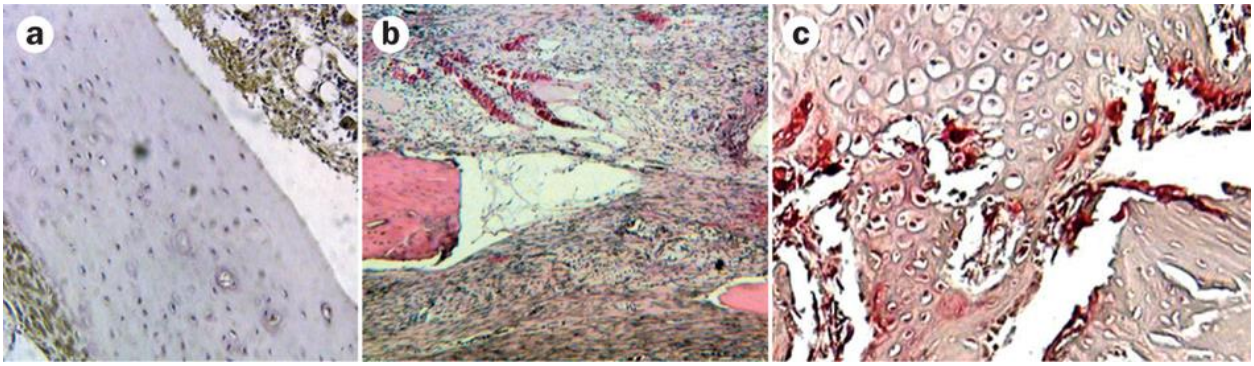


Figure 5. *Histology of secondary bone healing: inflammatory stage, late inflammatory and late osteochondral stage. (Einhorn and Gerstenfeld 2014) with permission*

1.3. Conditions affecting fracture healing

Several physiological, pathological and environmental conditions could negatively affect the process of fracture healing, such as diabetes, smoking and old age.

As we age, the rate of fracture healing declines (Skak and Jensen 1988). This could be the reason non-unions are considered a serious clinical condition (Nieminen, Nurmi et al. 1981). Several signalling pathways are involved in normal bone cell differentiation, disruption of any of these pathways could potentially affect normal osteoblastogenesis and change the fate of mesenchymal cells and osteochondroprogenitors **Figure *6*** . A reduction in the signaling pathway molecules

during the inflammatory phase has been suggested a key event leading to a reduction in the recruitment of chondroblast progenitors and in cartilage formation (Naik, Xie et al. 2009). Furthermore, a lack of normal upregulation of other signals (i.e. BMP-2 and Wnts) has been documented in aged mice during the maturation of chondrocytes and differentiation of osteoblasts (Meyer, Desai et al. 2006, Bajada, Marshall et al. 2009). A decline in the number of endothelial cells and lack of a fully sufficient newly formed blood vessels at the fracture site have also been proposed as the factors to impair bone healing in the elderly (Edelberg and Reed 2003, Brandes, Fleming et al. 2005).

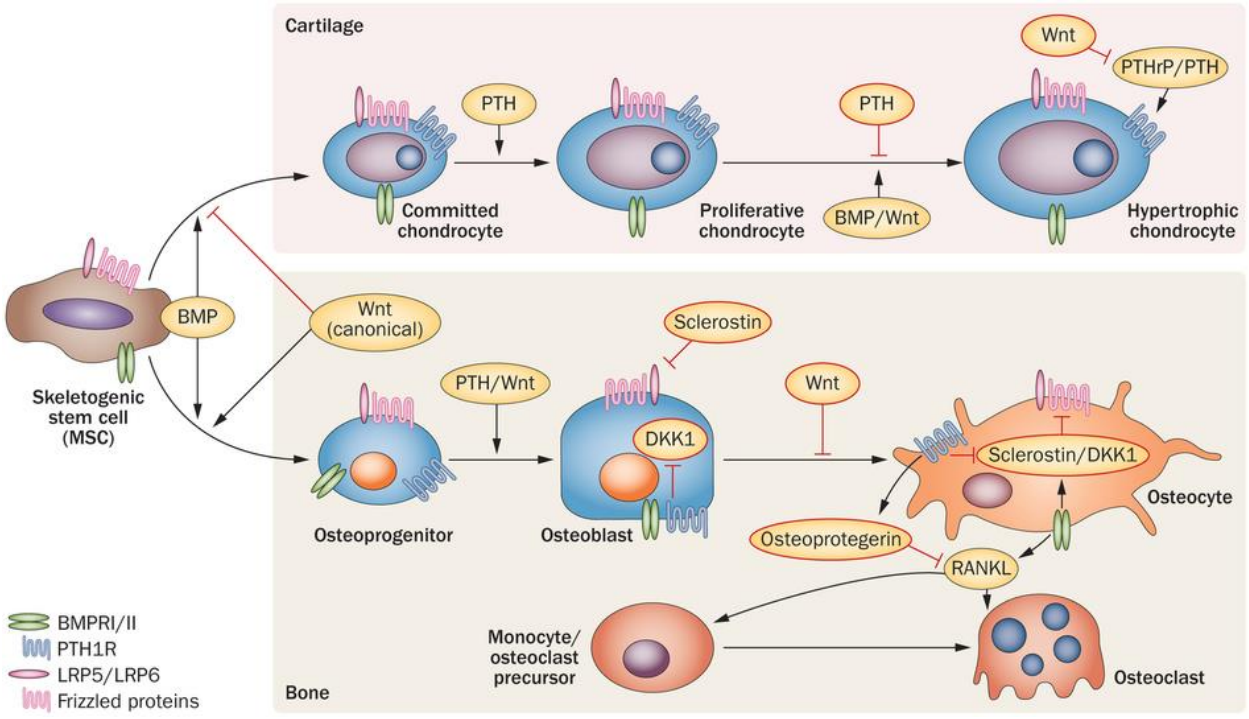


Figure 6. *Schematic pathways (Wnt, BMP, Smads, Dkk1, etc.) involved in the differentiation of mesenchymal progenitors to either chondroblasts or osteoblasts.* ***(Einhorn and Gerstenfeld 2014)*** *with permission*

Bone healing in individuals and animal models of diabetes type 1 is impaired. It is not known whether this is due to high glucose levels or low insulin in diabetes, however, insulin therapy to correct and normalize the levels of insulin and glucose in the animal models of diabetes, has rescued their poor fracture healing (Hough, Avioli et al. 1981, Kayal, Alblowi et al. 2009). The hypothesis that insulin has a direct effect on fracture healing has been supported by the research undertaken by Gandhi et al which showed local application of insulin to the site of fracture in diabetic rat could reverse the impaired bone repair (Gandhi, Beam et al. 2005). Although poor fracture healing has been documented clinically in diabetes type 2 in the obese population, the underlying molecular mechanism of such impairment is not clear yet (Khazai, Beck et al. 2009).

In the clinic, cigarette smokers have a lower rate of fracture healing of long bones compared to the non-smokers (Schmitz, Finnegan et al. 1999, Sloan, Hussain et al. 2010). It is the case for spinal fusion surgeries as well (Hadley and Reddy 1997). Like diabetes, the underlying mechanisms through which smoking affects bone repair are not fully understood. As potential targets during the healing process, mesenchymal cell recruitment and chondrogenesis have been proposed to be negatively impacted by cigarette smoking (Ueng, Lee et al. 1997, El-Zawawy, Gill et al. 2006). Nicotine in the cigarette smoke has been implicated to supress the fracture healing process, distraction osteogenesis and spinal fusion (Raikin, Landsman et al. 1998, Silcox, Boden et al. 1998, Ma, Zheng et al. 2007).

1.4. Clinical approach

In the context of bone regeneration and fracture repair, if the amount of bone loss is considerably large, particularly due to trauma, large tumor surgical excision, developmental anomalies or extensive concurrent infections, then bone tissue fails to heal on its own and therapeutic interventions are necessary. Such large bone defects are referred to as “critical size defects” (CSD) and are considered substantial clinical complications to manage in craniofacial and reconstructive orthopedic practice **Figure *7*** as the spontaneous process of healing does not occur (Alvira-Gonzalez, Sanchez-Garces et al. 2016, Ghadakzadeh, Mekhail et al. 2016).

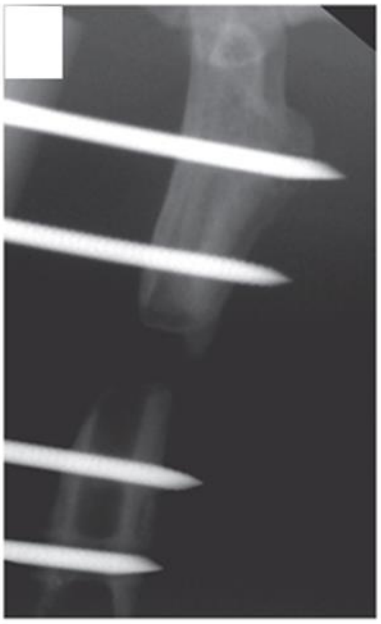


Figure 7. *Critical size defect in the rat femur (Evans and Huard 2015) with permission.*

There are different clinical approaches to treat CSDs including Ilizarov and Masquelet techniques or bone graft strategies. These techniques are described briefly here:

1.4.1. Ilizarov technique

Ilizarov technique has been invented by Dr. Gavril Ilizarov in 1960s. It involves a distractible external metal frame and acts based on the principles of distraction osteogenesis (Brunner, Kessler et al. 1990). **Figure 8**

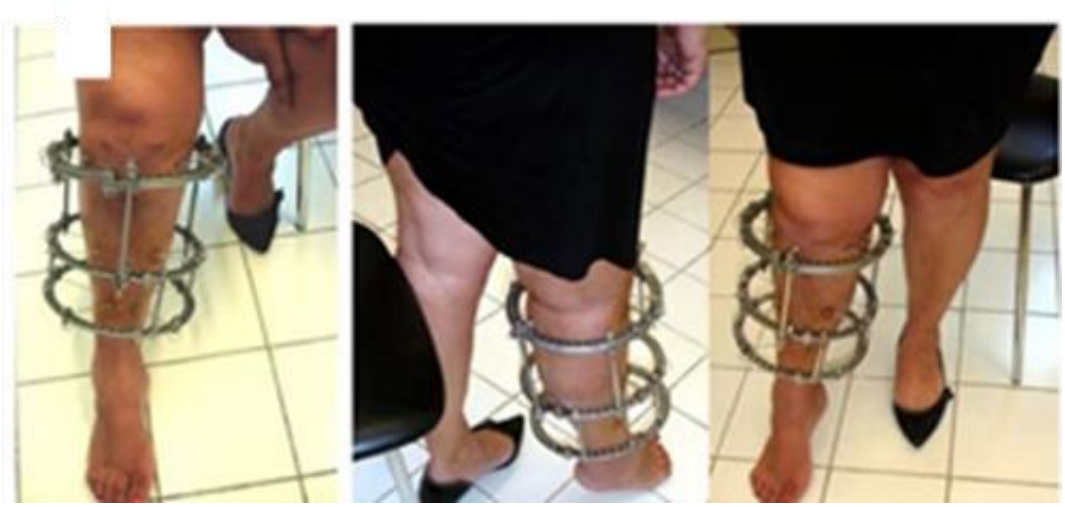


Figure 8. *Ilizarov technique for long bones (Grivas and Magnissalis 2011). with permission.*

Although Ilizarov method is commonly utilized in orthopaedic and reconstructive surgery, there are several downsides with this technique, warranting the need for alternative treatment approaches (Iacobellis, Berizzi et al. 2010).

The complications that could occur during limb lengthening using Ilizarov method include but are not limited to: neurologic injury, vascular injury, nonunion, premature consolidation, muscle contractures, joint luxation, axial deviation, delayed consolidation, pin site problems (i.e. infections and bleeding), re-fracture and hardware failure. Joint

stiffness could happen and remain as an everlasting difficulty. Severe pain, sleep disorders and psychological consequences are other problems arising throughout the lengthy healing process, particularly in more complicated scenarios (Paley 1990).

1.4.2. Masquelet technique

Masquelet et al. introduced a surgical technique which is a combination of induced membranes and cancellous autografts in 2000 (Masquelet, Fitoussi et al. 2000). **Figure 9** The actual bone grafting for CSDs is usually postponed for the time that the soft tissue healing has occurred, this allows elimination of the risk of infection, and prevents the resorption of the bony graft (McCall, Brokaw et al. 2010).

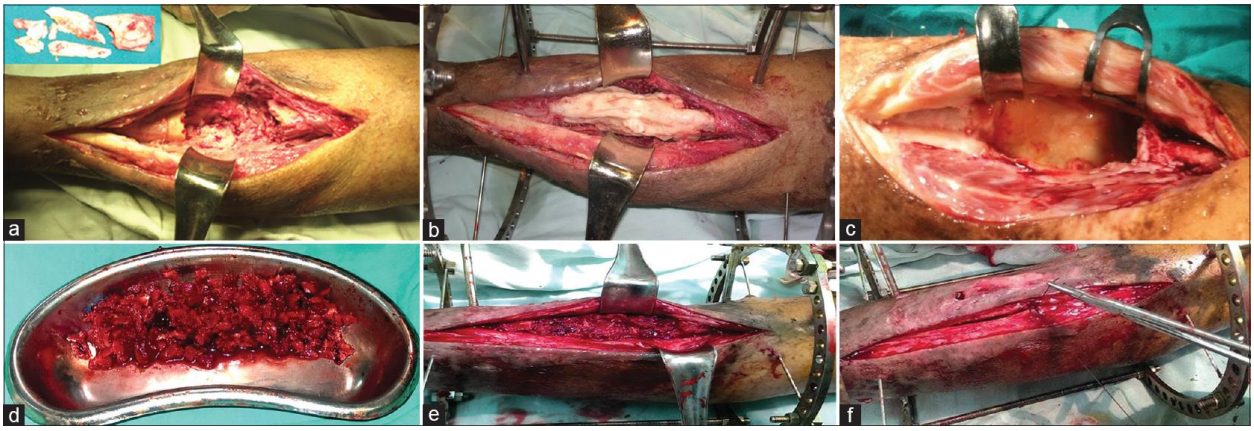


Figure 9. *Masquelet technique. Different stages of the procedure including the debridment of infected and necrotic bone to reach a healthy bleeding bed, filling of the defect with bone cement and in the second round of surgery, the cement is removed and the gap is filled with small pieces of cancellous bone graft. Adapted from (El-Alfy and Ali 2015) with permission.*

As described above, both these techniques are extremely complicated and require a great deal of experience to achieve successful results and to avoid the risk of nerve or vasculature damage. Both procedures are laborious and the complete term of treatment could last several months. Considering the amount of pain during these extensive and slow processes, patient compliance is a major factor determining the success rate (Mertens and Lammens 2001).

1.4.3. Bone graft

Several bone graft techniques have been introduced to manage CSDs, however, the gold standard treatment, which is most widely employed in orthopaedic practice, is autologous bone grafting (Sen and Miclau 2007, Polyzois, Stathopoulos et al. 2014). In this type of bone graft, the bone tissue is harvested from another site of the patient's skeleton (usually iliac crest) and transferred to the site of bone defect (Myeroff and Archdeacon 2011). As the donor is the patient, risks of immune response and tissue rejection is eliminated (Azi, Aprato et al. 2016).

Despite encouraging results, several morbidities are associated with this technique as a result of postoperative infections (Mostly at the donor site) and elongated hospitalization time. Additionally, the amount of available autologous bone from a patient is obviously limited, particularly in large defects requiring larger amount and also in pediatric practice where the overall amount of bone is limited (Ring, Allende et al. 2004). Considering the fact that an additional invasive surgical procedure is required to extract bone at the donor site (usually from the iliac crest) the patient is put

at greater risk to develop additional complications such as extensive pain, infection, bleeding, nerve or vessel injuries, etc. (Ebraheim, Elgafy et al. 2001, Patel, Watson et al. 2003).

1.5. Tissue engineering for bone repair

Tissue engineering holds enormous potential to present promising alternatives to traditional surgical approaches such as autologous bone grafts (Tare, Kanczler et al. 2010, Santos, Pandita et al. 2011). The core concept of any tissue engineering approach for bone repair involves the consideration of four main components (i.e., Diamond Concept): a “scaffolding biomaterial” to provide appropriate environment for the cells to grow in/on, involvement of “growth factors” (both osteoinductive and angiogenic) to stimulate tissue growth, the “cells” with potential osteoblastic differentiation fate and finally the mechanical environment including the forces applied to the site of defect and the degree to which the whole system is allowed to move (Giannoudis, Einhorn et al. 2007, Balmayor 2015). **Figure 10**

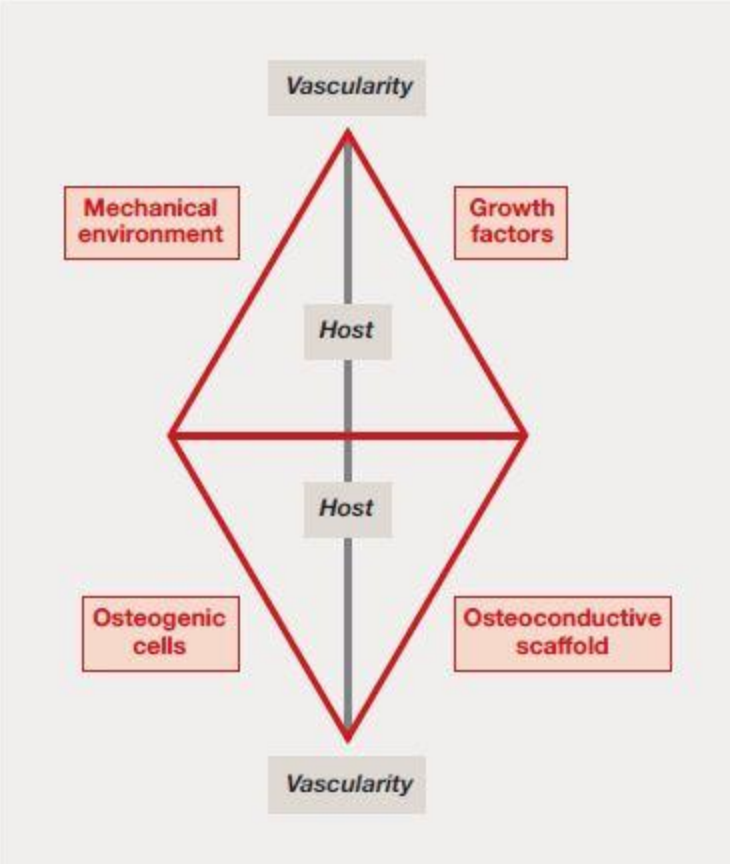


Figure 10. *The "diamond concept" of bone regeneration. Adapted from: Giannoudis et al. Injury. 2008;39(suppl 2):S5-S8. © 2008, Elsevier Ltd.* With permission.

In tissue engineering, stem cells, for instance, could be pluripotent, however, in order for them to differentiate to other cell types, they would need to interact with different growth factors through signaling pathways. Similar interactions occur between cells and the type of scaffold surfaces which dictate a certain direction in cell differentiation to either osteoblasts or chondroblasts, for instance. To mimic the natural bone growth, cells need a 3D structure to grow on; this is achieved by designing certain 3D scaffolds to prepare suitable environment for cells and their extra cellular matrix to adhere to and shape the desired engineered tissue (Sandor 2013).

Biomaterials are equally important in the design of efficient drug delivery systems (DDSs) with controlled release properties for growth factors at the exact site of injury over time. One of the most important advantages of controlled release properties is the capability of such systems to induce a therapeutic effect with significantly reduced concentrations of their cargo (i.e. the growth factors). This privilege leads to lower overall healthcare costs and eliminates the chances of development of unwanted side effects which are commonly associated with high doses of growth factors.

1.6. Growth factors for bone repair

Several growth factors have been recently employed in research and clinic to induce bone formation through different phases of bone repair. The areas of interest include fracture repair, mal-union and non-unions, spinal surgery, craniofacial and dental procedures (Balmayor 2015). Bone morphogenetic proteins (BMPs) are among the most widely tested growth factors for tissue engineering (Simpson, Mills et al. 2006, Aagaard and Rossi 2007). As described earlier, BMPs are key growth factors that are naturally secreted by different cell types during the healing process. They are capable of initiating a cascade of intracellular signalling pathways to stimulate downstream osteoblast-related downstream gene expression and protein secretion. Initial steps involve the interaction of BMPs with specific cell surface receptors in the skeletal tissue.

BMPs are able to signal through either canonical or non-canonical pathways. Here we briefly describe the canonical pathway, in which they initiate the signal cascade by binding to their specific receptors to shape a complex made up of two serine/threonine kinase receptors. Seven type I receptors and four type II receptors are

known for the TGF-β family of ligands. The mechanism of the formation of ligand induced signaling complex could be different for different BMPs. When this complex is formed, the type II receptor activates the type I receptor and this leads to the phosphorylation of Smad proteins which are direct downstream targets of the signal. Smads then form another complex which translocates into the nucleus to act as a transcription factor and regulates the expression of certain downstream genes within the TGF-β family (Wang, Green et al. 2014).

Basic and clinical research have led to the production of recombinant forms of BMP2 and BMP 7 (rhBMP2 and rhBMP7) which are commercially available for clinical use in several countries (Aagaard and Rossi 2007, Gautschi, Frey et al. 2007, Haidar, Hamdy et al. 2009, Haidar, Hamdy et al. 2009). Nonetheless, due to their extremely short half-life in the body, and their tendency to dilute quickly at the initial site of application, very large doses – usually thousands of times higher than physiological concentrations- are necessary for achieving a satisfactory and therapeutic outcome (Croteau, Rauch et al. 1999, Haidar, Hamdy et al. 2009). In addition to the unacceptably huge cost of these amounts of recombinant proteins, serious safety issues are expected for the recipients, including but not limited to drug toxicity, osteolysis, retrograde ejaculation, heterotopic ossification, seroma/hematoma, wound infection, and dysphagia (Haidar, Hamdy et al. 2009, Haidar, Hamdy et al. 2009, Evans 2010).

1.7. Clinical application of BMP for long bone fractures

Several previous studies, including recent systematic reviews of the literature have evaluated the effectiveness of the use of BMPs for the management of long bone

fractures and CSDs in comparison with the conventional surgical approaches, i.e. autologous bone graft as the orthopaedic gold standard.

Utilizing BMPs in the management of orthopaedic injuries, however, has been the matter of debate by several authors. BMPs encourage the recruitment of bone-forming cells to the area of defect and contribute to the healing. Several BMPs are known to have a key role in the proliferation and differentiation of osteoblast precursor cells, as a result, promoting bone formation (Krishnakumar, Roffi et al. 2017). There are reports showing that BMPs have a significant role in the regulation of all three major phases of fracture repair (inflammation, chondrogenic phase, and osteogenic phase) and vice versa; nevertheless, there are several mechanistic questions still unanswered (Cho, Gerstenfeld et al. 2002, Yu, Lieu et al. 2010, Krishnakumar, Roffi et al. 2017). The use of BMP for fracture healing purpose currently has a few FDA-approved indications (Ronga, Fagetti et al. 2013). At the beginning, the potentials of the effectiveness of BMP treatment were considered high, however, during the past years the union rate of fractured bones treated with BMPs was demonstrated to be similar with autografts (Blokhuis, Calori et al. 2013). The effectiveness of BMPs have been tested in several clinical trials with non-unions, fractures and osteonecrosis (Friedlaender, Perry et al. 2001, Govender, Csimma et al. 2002, Giannoudis and Tzioupis 2005, Swiontkowski, Aro et al. 2006, Calori, Tagliabue et al. 2008, Sun, Li et al. 2014). A very recent systematic review by Krishnakumar et al (Krishnakumar, Roffi et al. 2017) has been published where the authors have studied the clinical evidence on BMPs for the treatment of non-unions, fractures and osteonecrosis by reviewing the relevant clinical literature. They have summarized the potentials of effectiveness and of the

complications of BMP use, as well as the cost effectiveness of such treatments. In total, 44 articles published between 2000 and 2016 have been included in this study and their full texts were reviewed.

The review of literature relating to the use of BMPs for treatment of open fractures revealed: seven randomized clinical trials (RCTs), two comparative studies, only one case series, and five clinical case reports. Out of these 15 studies, 9 focused on fractures in tibial, one in the femur, 3 in humerus, and two in the forearm. These results have been summarized in **Table 1**.

Table 1. Summary of clinical trials on rhBMPs application in fractures

rhBMP	*Authors*	*Study*	*No of patients*	*Delivery*	*Dose*	*Commercial name*	*Site*	*F-up*	*Result*	*Adverse events*
rhBMP-2	Govender 2002 [12]	RCT	150 pts. IM 151 pts. IM + low dose rhBMP-2 149 pts. IM + high dose rhBMP-2	ACS	0.75 mg/ml Vs 1.5 mg/ml	Inductos®	Tibia	12 m	+ For high dose	None
	Jones 2006 [23]	RCT	15 pts. ABG 15 pts. allograft + rhBMP-2	ACS	1.5 mg/ml	Infuse®	Tibia	12 m	+	None
	Swiontkowski 2006 [16]	RCT	131 pts. Gustilo-Anderson III (66 + rhBMP-2)113 pts. IM (65 + rhBMP-2)	ACS	1.5 mg/ml	Infuse®	Tibia	12 m	+	None
	Schwartz 2008 [24]	Case report	1 pt. rhBMP-2 + Mastergraft®	ACS	1.5 mg/ml	Infuse®	Ulna	22 m	+	None
	Boraiah 2009 [25]	Comparative study	23 pts. allograft 17 pts. allograft + rhBMP-2	ACS	1.5 mg/ml	Infuse®	Tibia	18 m	−	Heterotopic bone formation in rhBMP-2 group (10/17)

	Aro 2011 [26]	RCT	136 pts. IM 139 pts. IM +rhBMP-2	ACS	1.5 mg/ml	Infuse®	Tibia	5 m	=	More infections (19%) in rhBMP-2 Other complications peripheral oedema (26%), heterotopic ossification (26%), pain (63%)
	Capo 2011 [27]	Case report	1 pt ABG + rhBMP-2	ACS	1.5 mg/ml	Infuse®	Radius and Ulna	9 m	+	None
	Baltzer 2012 [28]	Case report	1 pt. rhBMP-2 injection	None	2 mg/ml	Inductos®	Femoral neck	2 m	+	None
	Julka 2012 [29]	Case report	1 pt. rhBMP-2	ACS	1.5 mg/ml	Infuse®	Humerus	4–5 m	+	Heterotopic bone formation, secondary vein compression, massive inflammatory reaction
	Lyon 2013 [30]	RCT	62 pts. IM 122 pts. IM + low dose rhBMP-2 125 pts. IM + high dose rhBMP-2 60 pts. IM + buffer	CPM	1 mg/ml Vs 2 mg/ml	N/A	Tibia	13 m	−	Severe-treatment adverse events more frequently in rhBMP group (25%)
rhBMP-7	Maniscalco 2002 [31]	RCT	7 pts. EF 7 pts. EF + rhBMP-7	ACS	3.3 mg/ml	OP-1 Stryker	Tibia	6 m	=	None
	Mc Kee 2005 [32]	RCT	62 pts. IM 62 pts. IM + rhBMP-7	ACS	N/A	N/A	Tibia	6 m	+	None

	Risitiniemi 2007 [33]	Comparative study	20 pts. EF 20 pts. EF + rhBMP-7	Coll I powder	3.3 mg/ml	Osigraft®	Tibia	20 m	+	Heterotopic ossification in rhBMP group, no symptoms
rhBMP-2 and 7	Axelrad 2008 [34]	Case series	3 pts. rhBMP-7 + thrombin pouch 1 pt. rhBMP-2	Coll I Powder and ACS	3.3 mg/ml rhBMP-7 1.5 mg/ml rhBMP-2	Infuse® OP-1 Stryker	Humerus	6–12 m	−	4/4 pts. developed heterotopic bone formation

Abbreviations: RCT: randomized control trial, BMP-2: bone morphogenetic protein 2, BMP-7: Bone Morphogenetic protein 7. ABG: autologous bone grafting, IM: intramedullary reaming, EF: external fixator, ACS: absorbable collagen sponge, CPM: calcium phosphate matrix, Coll I: collagen type I; N/A: information not available, +: positive response for BMPs, −: negative response for BMPs (Table adapted from (Krishnakumar, Roffi et al. 2017) With permission with minor modifications)

1.7.1. rhBMP-2 and fractures

The randomized clinical trial on BMP-2 and the evaluation in the surgical treatment of tibial trauma (BESTT) performed in 2002 (Govender, Csimma et al. 2002) examined the safeness and efficiency of commercial rhBMP-2 in the treatment of tibial fractures in 450 cases. The patients were randomized and received treatment in three groups: 151 patients were treated with a dose of 0.75 mg/ml of rhBMP-2 contained in a commercially available Absorbable Collagen Sponge (ACS, Helistat; Integra LifeScience, Plainsboro, New Jersey), 149 patients received double the amount of rhBMP-2 in ACS, and in the last group, 150 patients did not receive any rhBMP in addition to the standard surgical procedure. One year post-surgery, the second group (rhBMP-2 at the dosage of 1.5 mg/ml) showed better results in quickening the healing of tibial fractures with an acceptable safety profile. In the assessment of the efficacy of the treatment, factors such as: the occurrence of additional interventions necessary, the infection frequency, and the amount of invasive procedures necessary were comparatively less. Another study in 2006 (Swiontkowski, Aro et al. 2006) on the above participants in addition to another study group in the US, was performed with regards to the type of fractures and utilizing 1.5 mg/ml dose of rhBMP-2. Subgroup I contained 131 patients with Gustilo-Anderson (a classification which indicates the severity of the fracture) type IIIA or IIIB tibial fractures and Subgroup II: 113 patients with Gustilo-Anderson type I-type IIIB tibial fractures. The analysis of the results showed that rhBMP-2 significantly reduced the rate of future interventions (i.e. bone grafting) in a one year follow up duration.

Jones et al. evaluated the effectiveness of rhBMP-2 in 30 patients with tibial fractures considerable bone loss (Jones, Bucholz et al. 2006): 15 patients were treated with standard orthopedic procedure, autologous bone graft (ABG), and another group of 15 patients with rhBMP-2 in addition to allograft. At one year timepoint, the authors did not observe any significant difference in the rate of union or bone repair, or even in quality of life improvement scores, signifying that the addition of rhBMP-2 did not put the patients at a greater risk of non-union or quality of life impairment. A large RCT was performed in 2011 by Aro et al. to evaluate the effect of addition of rhBMP-2 in the treatment of tibial fractures (Aro, Govender et al. 2011). Of the 300 patients recruited for the study, 136 received IM fixation surgery and 139 underwent IM nailing combined with rhBMP-2. No significant improvement in fracture healing was observed in the rhBMP-2 treated group compared to the other group, however, the rate of post-surgical complications was lower in this group except for infection which occurred more frequently in the BMP treated group. Also, Lyon et al. (Lyon, Scheele et al. 2013) found no significant difference between groups treated with or without rhBMP-2 for their tibial fractures: 62 patients were treated with IM fixation, 122 patients with IM fixation and 1 mg/ml of rhBMP-2 (low concentration) in a calcium phosphate matrix (CPM), 125 patients with IM fixation and 2 mg/ml (high concentration) of rhBMP-2/CPM injected at the site of bone defect, and 60 more patients IM fixation with sham/CPM injection. A follow-up for 1 year and 1 month, showed no improvement in the time to union seen in radiographic of the test groups. Similarly, no significant improvement was observed in the full-weight bearing without pain in both rhBMP-2 treatment groups. However, more

side effects (such as heterotopic ossification or tissue edema) compared with the control group.

1.7.2. rhBMP-7 and fractures

In 2002, Maniscalco et al. (Maniscalco, Gambera et al. 2002) studied and reported the outcome of rhBMP-7 use in tibial fractures in 14 patients. In this study, 7 patients in each group received external fixation (EF) with or without rhBMP-7 (7 EF and seven patients received additional rhBMP-7). Evaluating the patients after 6 months, no complication was reported I neither groups, all patients in both groups showed bony bridging, with similar duration to treat. In a similar study by the Canadian Orthopaedic Trauma Society in 2005 (McKee 2005), rhBMP-7 was tested in 124 patients with tibial shaft fractures. There were two groups of patients with 62 patients in the standard treatment IM procedure and another 62 patients in the rhBMP-7 treatment group. Six months later, the results showed a significantly lower incidence of non-union, delayed union. Also, post surgical intervention rate and adverse events rate was significantly lower in the rhBMP-7 treatment group.

1.8. BMPs and non-unions

Three RCTs, 4 comparative studies, 15 case series, and 3 case reports were identified utilizing BMPs for the treatment of non-unions, occurring in different anatomical locations, such as femur, tibia (7 studies), humerus (4 studies) and ulna (1 study).

Table 2.

The group of Friedlaender et al. (Friedlaender, Perry et al. 2001) utilized rhBMP-7 for the surgical treatment of tibial non-unions (IM fixation) in 122 patients which were divided into 61 patients in the test group 61 patients in the control group. At 9 months and 2 year intervals the patients were examined, there was no difference between groups and all the patients healed completely. Calori et al. (Calori, D'Avino et al. 2006) in 2006 explored the safety and efficacy of rhBMP-7 in the treatment of non-unions, they studied this biological treatment was tested in different anatomical body sites of 29 patients, 16 of them received rhBMP-7 in the bone graft. The results from this study revealed that rhBMP-7 were apparently more effective, and induced better healing with less failure rate. The researchers performed a larger cohort on 120 patients, 60 patients in each arm of the atudy, and confirmed these results in 2008 (Calori, Tagliabue et al. 2008):. The rhBMP-7 group healed faster confirmed by imaging.

1.9. Complications

Some safety concerns have been associated with the use of BMPs as several negative events were reported. One of the worst complications was reported in patients undergoing spine surgery and rhBMP-2 was utilized. In these patients, vertebral osteolysis, graft rejection and movement were observed. In some patients, haematoma and ectopic bone was formed and neurological symptoms (i.e. numbness) were recorded (Garrett, Kakarla et al. 2010, Hoffmann, Jones et al. 2013, Cole, Veeravagu et al. 2014). In 2011, FDA released a warning in regard to the off-label use of commercial

rhBMP-2 (from Medtronic) as a result of the above mentioned complications and negative events reported (Carragee, Hurwitz et al. 2011, Faundez, Tournier et al. 2016).

There is evidence of medical issues associated with BMP use in the literature. These studies show that heterotopic ossification has been the most reported complication in patients with fractures (Axelrad, Steen et al. 2008, Boraiah, Paul et al. 2009, Julka, Shah et al. 2012), non-unions (Bong, Capla et al. 2005, Wysocki and Cohen 2007) , and osteonecrosis (Papanagiotou, Malizos et al. 2014) for whom BMP was utilized as part of their treatment plan. Furthermore, other complications remained associated with BMP use, such as: infections (Aro, Govender et al. 2011, Ollivier, Gay et al. 2015), new or augmented pain (Aro, Govender et al. 2011).

Lastly, one study reported a systemic reaction to BMPs, associated with increased antibody levels against BMP-2, however, there was no association between this negative event and the treatment efficacy of BMP in fracture healing (Aro, Govender et al. 2011). No other allergic reaction or systemic event has been reported in the literature.

It is worth to mention that other therapeutic agents such as small molecules (eg, phenamil, purmorphamine) (Lo, Ashe et al. 2012), synthetic peptides (Choi, Lee et al. 2010), or other off label drugs (eg, statins, melatonin) have been explored in addition to BMPs (Aagaard and Rossi 2007, Lo, Ulery et al. 2014, Balmayor 2015, Lo, Kan et al. 2016) but are out of the context of this thesis.

Table 2. Summary of clinical trials involving rhBMPs application for non-union

RHBMP	*AUTHORS*	*STUDY*	*NO OF PATIENTS*	*DELIVERY*	*DOSE*	*COMMERCIAL NAME*	*SITE*	*F-UP*	*RESULT*	*ADVERSE EVENTS*
RHBMP-2	Crawford 2009 [35]	Case series	9 pts. rhBMP-2	N/A	N/A	N/A	Humerus	N/A	+	None
	Tressler 2011 [36]	Comparative study	74 pts. ABG 19 pts. allograft + rhBMP-2	ACS	12 mg/cm2	Infuse®	Multiple locations	20 m	+	None
	Desai 2010 [37]	Case series	9 pts. ABG + rhBMP-2	ACS	1.5 mg/ml	Infuse®	Tibia	5 m	+	None
RHBMP-7	Friedlaender 2001 [14]	RCT	61 pts. ABG 61 pts. rhBMP −7	Coll I powder	3.3 mg/ml	OP-1 Stryker	Tibia	24 m	+	Higher rate of osteomielytis in ABG group (13/61)
	Pecina 2003 [38]	Case report	1 pt. rhBMP-7 + BMAC	Coll I powder	3.3 mg/ml	OP-1 Stryker	Tibia	18 m	+	None
	Kujala 2004 [39]	Case series	5 pts. coral frame + rhBMP-2 (autografts if required)	Collagen carrier	2–5 mg/cm3	None	Ulna	14 m	+	None
	Giannoudis 2005 [15]	Case series	395 pts. rhBMP-7 (autografts if required)	Coll I powder	3.5 mg/ml	OP-1 Stryker	Multiple locations	15.3 m	+	None
	Bong 2005 [40]	Case series	23 pts. rhBMP-7 (autografts if required)	Coll I powder	3.5 mg/ml	Osigraft®	Humerus	9 m	+	1/23 pts. heterotopic ossification and nerve complications

Dimitriou 2005 [41]	Case series	25 pts. rhBMP-7 (autografts if required)	Coll I powder	3.5 mg/ml	OP-1 Stryker	Multiple locations	15.3 m	+	None
Calori 2006 [42]	RCT	16 pts. rhBMP-7 13 pts. PRP	Coll I powder	3.5 mg/ml	Osigraft®	Multiple locations	18.8 m	+	None
Ronga 2006 [43]	Case series	105 pts. rhBMP-7 (autografts if required)	Coll I powder	3.5 mg/ml	Osigraft®	Multiple locations	29.2 m	+	None
Wysocki 2007 [44]	Case report	1 pt. rhBMP-7 (autografts if required)	Coll I powder	3.5 mg/ml	OP-1 Stryker	Humerus	2 m	–	Heterotopic ossification of the triceps muscles
Kanakaris 2008 [45]	Case series	68 pts. rhBMP-7 (autografts if required)	Coll I powder	3.5 mg/ml	Osigraft®	Tibia	18 m	+	None
Desmyter 2008 [46]	Case series	62 pts. rhBMP-7 (autografts if required)	Coll I powder	3.5 mg/ml	OP-1 Stryker	Tibia	12 m	+	None
Calori 2008 [13]	RCT	60 pts. rhBMP-7 60 pts. PRP	Coll I powder	3.5 mg/ml	Osigraft®	Multiple locations	12 m	+	None
Giannoudis 2009 [47]	Case series	45 pts. ABG + rhBMP-7	Coll I powder	3.5 mg/ml	Osigraft®	Multiple locations	24 m	+	None
Zimmermann 2009 [48]	Comparative study	82 pts. ABG 26 pts. rhBMP-7	Coll I powder	3.5 mg/ml	Osigraft®	Tibia	12 m	+	None
Kanakaris 2009 [49]	Case series	30 pts. rhBMP-7 (autografts if required)	Coll I powder	3.5 mg/ml	Osigraft®	Femur	24 m	+	None
Moghaddam 2010 [50]	Case series	54 pts. rhBMP-7 (autografts if required)	ACS	3.5 mg/ml	Osigraft®	Multiple locations	6 m	+	None
O'hEireamhoin 2011 [51]	Case series	13 pts. rhBMP-7	Coll I powder	3.5 mg/ml	Osigraft®	Multiple locations	9 m	+	None

	Papanna 2012 [52]	Case series	52 pts. rhBMP-7 (autografts if required)	Coll I powder	3.5 mg/ml	Osigraft®	Multiple locations	12 m	+	1/52 pts. developed distal tibiofibular synostosis
	Murena 2014 [53]	Case report	2 pts. allograft +BMAC + rhBMP-7	Coll I powder	3.5 mg/ml	Osigraft®	Humerus	12 m	+	None
	Calori 2015 [54]	Comparative study	44 pts. ABG 44 pts. rhBMP-7	Coll I powder	3.5 mg/ml	Osigraft®	Multiple locations	9 m	+	None
	Ollivier 2015 [55]	Case series	20 pts. rCPBS + rhBMP-7	Coll I powder	3.5 mg/ml	Osigraft®	Tibia	14 m	+	1/24 infection
RHBMP 2 VS 7	Conway 2014 [56]	Comparative study	112 pts. ABG + rhBMP-2 63 pts. ABG + rhBMP-7	Coll I powder and ACS	1.5 mg/ml 3.3 mg/ml	Infuse® Osigraft®	Multiple locations	32 m	BMP-2 > 7	None

Table adapted from (Krishnakumar, Roffi et al. 2017) With permission with minor modifications

1.10. Gene Therapy

“Similar to delivery of growth factors, gene therapy aims to deliver genetic material in order to induce and stimulate new bone formation at the site of bone defects. This could be achieved by introducing genes to bone tissue with properties to either reduce bone resorption or to enhance the infiltration and proliferation of osteoblast precursors and cell differentiation toward an osteogenic lineage. Gene therapy methods explored for bone tissue engineering include vector-mediated ex vivo and *in vivo* DNA transfection of the cells. A variety of vectors and genes have been extensively explored for gene therapy in bone, detailed description of such methods is beyond the scope of this thesis, but could be found in other topic-specific reviews (Evans 2012, Balmayor and van Griensven 2015, Evans and Huard 2015).

1.10.1. RNA interference (RNAi)

RNA interference (RNAi) is an innovative biological mechanism that reduces the gene expression, characteristically by destruction of the transcript product (i.e. messenger RNA: mRNA) via different known processes. Two types of small RNA are fundamental to RNAi: micro RNA (miRNA) and small interfering RNA (siRNA). Natively expressed pri-mRNA molecules are short hairpin structures encoded by nuclear DNA and about 61 nucleotides (nt) long. These molecules are further processed in the nucleus to form pre-miRNAs and then exported to the cytoplasm. Pre-miRNAs are identified by a protein complex called Dicer which cleaves them to shorter miRNAs with two strands of non-perfect complementarity. Double-stranded miRNA is detected by Argonaute 2 (AGO2) and integrated into RNA-induced silencing complex (RISC). The

guide strand of miRNA guides this complex and binds the target mRNA with partial complementary, leading to mRNA translation repression.

Similarly, endogenous or exogenously introduced long double stranded RNA (dsRNA) is also identified by Dicer which cleaves the dsRNA to 20-25 nucleotide siRNAs, with perfect complementarity (Agrawal, Dasaradhi et al. 2003, Dykxhoorn, Novina et al. 2003). Then a single strand of siRNA, complexes with RISC and recognizes homologous mRNA substrates matched exactly with the siRNA sequence. RISC-siRNA complex mediates the cleavage of the mRNA to smaller pieces. Subsequently, the accumulation of mRNA in the cytosol would be reduced and this leads to a down-regulation of the target gene expression (Fagard and Vaucheret 2000, Bender 2001, Bernstein, Caudy et al. 2001, Elbashir, Martinez et al. 2001). The simplified series of these events are shown schematically in **Figure *11***. In addition to the naturally occurring intracellular miRNA and siRNA, the synthetic form of these molecules could be introduced to the cells exogenously (tenOever 2013, Arroyo, Gallichotte et al. 2014). There are several excellent reviews on this topic which are suggested for further reading (Bernstein, Denli et al. 2001, Hammond, Caudy et al. 2001, Sharp 2001, Ozcan, Ozpolat et al. 2015, Wittrup and Lieberman 2015).”

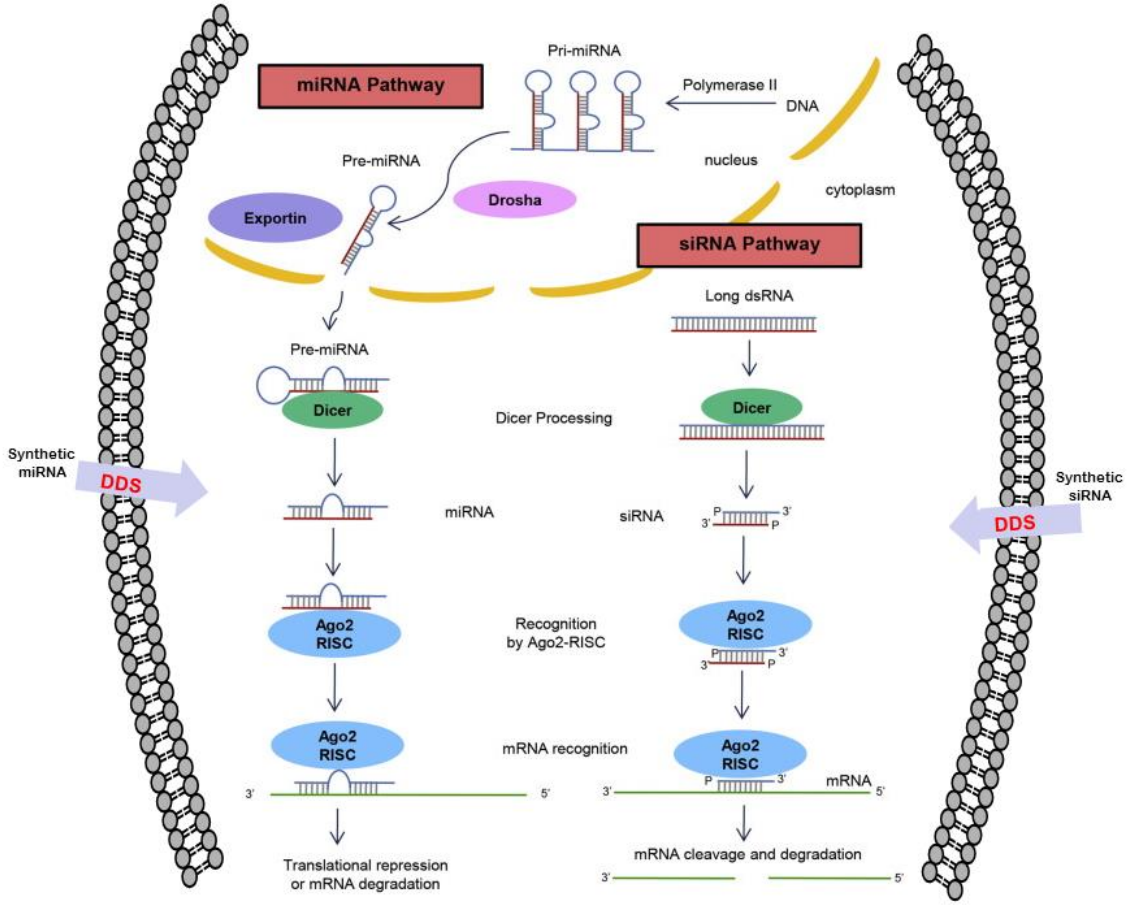


Figure 11. *Schematic mechanism of RNAi. (Dykxhoorn, Novina et al. 2003) siRNA: Transcribed or exogenously introduced long dsRNA is detected by a protein complex called Dicer, which cleaves it into multiple siRNAs then loaded into the RISC. One strand of siRNA is cleaved by AGO2, a component of RISC, the remaining strand (guide strand) guides the active RISC to recognize its target mRNA with exact complementarity. The RISC-siRNA complex mediates the cleavage and degradation of the mRNA and results in gene silencing. miRNA: Nuclear transcribed pri-miRNAs are cleaved by Drosha and form pre-miRNAs, which are later exported to the cytoplasm by Exportin. Pre-miRNA is detected and further processed by Dicer to miRNA, with two strands of imperfect complementarity. The miRNA is detected by AGO2 and is loaded*

into the RISC, where the passenger strand is discarded and the guide strand of miRNA guides this complex to bind the target mRNA with partial complementarity, leading to mRNA translation repression, degradation, or cleavage. Artificial siRNA or miRNA could be introduced directly to the cell via DDS to load into RISC for RNAi. Adapted from Ref. 27 with minor modifications. DDS = Drug Delivery System; dsRNA = double-stranded RNA; RISC = RNA-induced silencing complex.

"Short hairpin RNA is synthesized intracellularly by production of vector-mediated DNA (Paddison, Caudy et al. 2002, Moore, Guthrie et al. 2010). The structure of shRNA consists of two complementary 19-22 bp RNA sequences which are linked to each other by a non-complementary sequence of 4-11 nucleotides, forming a short loop, similar to the hairpin structure present in the natural miRNA (Moore, Guthrie et al. 2010). Expression of shRNA is attained using plasmids delivered to cells by viral or bacterial vectors (Davidson and McCray 2011). In addition to the unfavorable type of vectors (i.e. viral or bacterial) for future clinical applications, shRNA has been associated with a high risk of over-expression and toxicity in the cells (Borel, Kay et al. 2014).

Silencing property of siRNAs was initially revealed by Andrew Fire et al in 1998 (Fire, Xu et al. 1998) and has since been developing as an innovative approach to down-regulate target gene expression (McBride, Boudreau et al. 2008). RNAi, particularly siRNAs have been utilized as investigational tools to understand the biological role of specific genes by observing the consequences of knocking down that gene *in vitro* or *in vivo*. Similar to other tissues, bone-specific pathways and genes have been studied using this technique in several studies (Singhatanadgit, Salih et al. 2008, Song, Krause et al. 2010, de Gorter, van Dinther et al. 2011, Levi, Hyun et al. 2011,

Tiaden, Breiden et al. 2012, Kong, Liu et al. 2013, Kook, Jeon et al. 2013, Xu, Yu et al. 2013, Chang, Xiu et al. 2014, Du, Wang et al. 2014, Guerit, Brondello et al. 2014, Khaddam, Huet et al. 2014, Kook, Lim et al. 2014, Lee, Kim et al. 2014, Oh, Kim et al. 2014, Ozeki, Kawai et al. 2014, Shih, Hwang et al. 2014, Yan, Zhang et al. 2014, Yeh, Yang et al. 2014, Cho, Jue et al. 2015, Deng, Liu et al. 2015, Someya, Fujiwara et al. 2015, Son, Yang et al. 2015, Zhang, Liu et al. 2015).

However, recent applications of siRNA have gone beyond investigatory studies and into field of therapeutics (Kanasty, Dorkin et al. 2013). The ability to knockdown any gene of interest by knowing the mRNA sequence alone provides a cheap and robust technique for treating a wide range of diseases. In the case of bone regeneration, siRNA that can knockdown the expression of BMP inhibitors such as Noggin and Chordin has been widely explored as a means of enhancing bone formation. The major challenge, however, is the ability to deliver the siRNA into the cytoplasm of target cells and to promote the successful formation of the RISC complex (Gavrilov and Saltzman 2012). In order to overcome this issue, various drug delivery systems were developed in order to preserve the siRNA in the extracellular environment, promote its uptake by the target cells usually via endocytosis, and preserve it from enzymatic degradation within the cytoplasm. In this study, we provide a comprehensive analysis of the different siRNA delivery strategies for enhancing bone regeneration.

1.11. siRNA Drug Delivery Systems (DDS) for Bone Regeneration

There are two major categories of DDS that have been used for successfully delivering siRNA into osteoblastic cells, namely polymer- and lipid-based systems.

Polymers are long chains of repeating chemical units that can be modified to achieve a wide range of physicochemical properties. On the other hand, most lipid molecules used in DDS design are amphiphilic in nature, which means they contain hydrophilic and hydrophobic regions that allow them to form spherical vesicular structures in an aqueous environment. Both polymers and lipids are versatile and can be rationally designed to fabricate DDS that provide targeted and controlled delivery of therapeutic agents. Viral DDS have also been explored, mostly for *in vitro* studies and cell transfection, however, there are potential toxicity concerns associated with the use of live viruses, hindering their safe application in human. Most of studies reviewed, utilized either polymer or lipid-based systems, therefore, the focus of current paper will be on these systems.

1.11.1. Polymer-based DDS for siRNA delivery

Synthetic and natural polymers have been used for the past 50 years to fabricate a multitude of micro- and nanoparticles that can act as DDS for proteins, small molecules, and genetic material (Liechty, Kryscio et al. 2010). Conjugating moieties such as antibodies, short peptide sequences and aptamers have also been explored to improve DDS targeting to desired tissues once administered *in vivo*. Moreover, strategies used to overcome physiological barriers and improve intracellular uptake of polymer-siRNA systems have been thoroughly discussed in two excellent reviews (Whitehead, Langer et al. 2009, Liechty, Kryscio et al. 2010).

Y. Zhang et al. explored the use of a nanoparticle system fabricated from the synthetic copolymer, N-(2-hydroxypropyl) methacrylamide (HPMA) and conjugated with

poly-aspartic acid sequence and Alendronate (a bisphosphonate) for specific targeting to bone tissue (Zhang, Wei et al. 2014). HPMA is a highly hydrophilic polymer that is non-immunogenic and improves blood circulation of therapeutic agents, and was therefore explored as a promising DDS for siRNA (Ulbrich and Subr 2010). Moreover, the octa-aspartic acid sequence (D-Asp8) and bisphosphonates have been shown to target bone tissue since they recognize and specifically bind to hydroxyapatite (Wang, Sima et al. 2006). Semaphorins are molecules involved in cell-cell communication between osteoblasts and osteoclasts. Semaphorin 4d (Sema4d) specifically, has been shown to be released by osteoclasts in order to repel osteoblasts and reduce mineralization (Zhang, Wei et al. 2015). Therefore, gene silencing of Sema4d was explored as a treatment of osteoporosis in the mandibular alveolar bones of osteoporotic mouse models. The authors demonstrated that systemic administration of this bone-specific system lead to targeting high bone turn over sites including mandible alveolar region, peaking at four hours after intravenous injection of the drug. They also showed that the treatment interferes with Sema4d in the target areas *in vivo* and decreases the bone loss resulted from osteoporosis by anabolic effects on bone regeneration without affecting osteoclast count **Figure *12***A. The latter finding is advantageous over the use bisphosphonates, since bisphosphonates deteriorate osteoclasts and therefore can potentially affect natural bone turnover.

Poly(lactic-co-glycolic) acid (PLGA), a synthetic copolymer, has also been explored as a viable DDS for the delivery of siRNA. PLGA is biocompatible, biodegradable and FDA-approved for use in therapeutic devices (Makadia and Siegel 2011). Liu Hong et al. (Hong, Wei et al. 2012) fabricated PLGA micro particles and were

able to encapsulate siRNA and provide a controlled release *in vitro* for up to 40 days. siRNA against glucocorticoid receptors (GR) was used in order to reduce endogenous glucocorticoid (GC) activity in human MSCs. Elevated levels of GC were shown to reduce the ability for MSCs to proliferate by causing them to readily differentiate. Therefore, by silencing the expression of GR and reducing the effect of GC, MSCs were hypothesized to proliferate more readily and preferentially differentiate into the osteogenic lineage. The siRNA mediated gene silencing resulted in significant higher proliferation rates and differentiation towards osteoblasts, with lower adipogenic differentiation. The authors concluded that the PLGA micro particle system was a promising DDS for the transfection and delivery of siRNA to MSCs. Another study explored the use of PLGA micro particles encapsulating RANK siRNA in order to reduce bone resorption (Wang, Tran et al. 2012). RANK receptors on the surface of osteoclasts activate osteoclastic gene expression, which increases bone resorption. Therefore, silencing RANK was hypothesized to reduce bone resorption. The authors used the PLGA-siRNA microparticle system mixed with commercially available bone cement as a platform to seed murine cells on the surface. Although this study did not show any effect of this strategy on the function of osteoclasts and/or bone resorption, the authors demonstrated good bioactivity of the system and effective siRNA transfection of cells followed by inhibition of progression toward an osteoclastic phenotype **Figure *12***B."

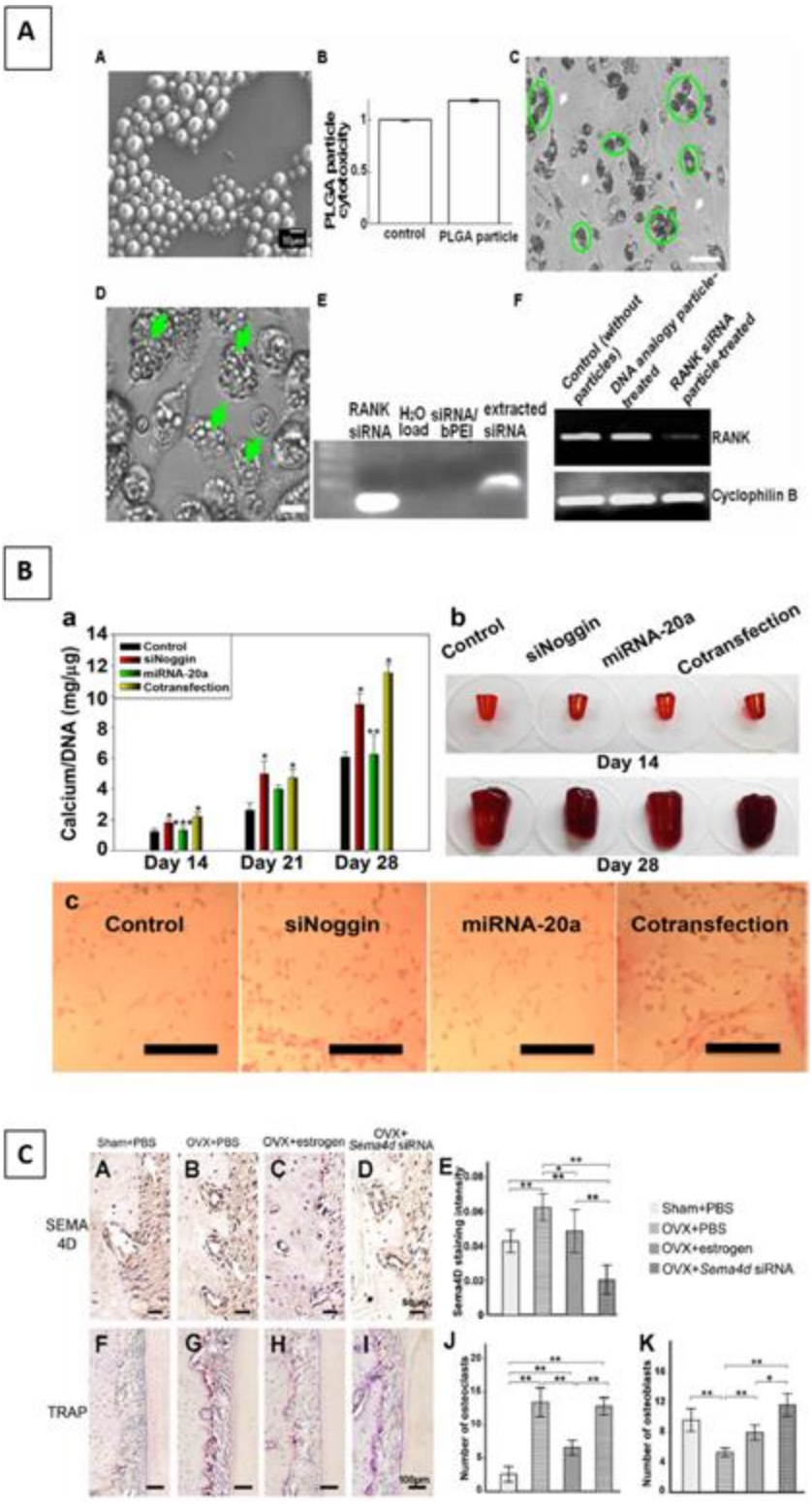


Figure 12. *(*A*) In vitro only: Encapsulation of siRNA in PLGA microparticles, physical, chemical, and functional properties of the microparticles (Wang, Tran et al. 2012) (*B*) In*

*vitro only: Alizarin red staining of hydrogels. Calcium content measurement in the hydrogels containing Noggin-siRNA or miRNA showing increased calcium content compared to the control group, over time (Nguyen, Jeon et al. 2014). (*C*) In vivo only: Representative immunohistological study of the intermolar regions in mice treated either with Sema4d siRNA or control: osteoblast and osteoclast numbers and* Sema4d *gene expression and knockdown (Zhang, Wei et al. 2014).*

"In addition to the use of polymer-based particles, 3D hydrogels have also been explored as DDS of siRNA for transfecting encapsulated cells. Minh K. Nguyen et al. (Nguyen, Jeon et al. 2014) fabricated 3D polyethylene glycol (PEG) scaffolds for localized and sustained delivery of siRNA to differentiate encapsulated human MSCs within the hydrogel network. siRNA was first complexed with polyethylenimine (PEI) – a highly cationic polymer – prior to incorporating in the hydrogel. The authors showed an enhanced osteoblastic differentiation of the human MSCs using this hydrogel as a result of prolonged delivery of Noggin siRNA or miRNA-20a (known to down regulate the expression of peroxisome proliferator-activated receptor gamma (PPAR-γ), a negative regulator of BMP-2). This study demonstrated interesting biomaterial methodology and approach, however, hMSCs from only 1-2 donors were examined **Figure *12***C.

Atelocollagen is another 3D gel system that was developed for siRNA delivery (Minakuchi, Takeshita et al. 2004). Atelocollagen is a decomposition product of type I collagen that is positively charged and thus can complex with siRNA (Minakuchi, Takeshita et al. 2004). Yohei Kawakami et al. (Kawakami, Ii et al. 2013) used atelocollagen to complex siRNA against Lnk (an inhibitory signaling molecule for stem cell renewal) in order to accelerate fracture healing. Primary mouse bone marrow cells

and osteoblasts were transfected by siRNA against Lnk using lipofection. For the fracture model experiments, the siRNA was administered to the site of fracture in a 3D Atelocollagen gel, which underwent thermally-induced gelation once injected *in vivo* (37 °C). This strategy was reported to contribute a favorable environment for fracture repair by promoting both angiogenesis and osteogenesis and resulted in enhanced recovery from facture **Figure 13**A."

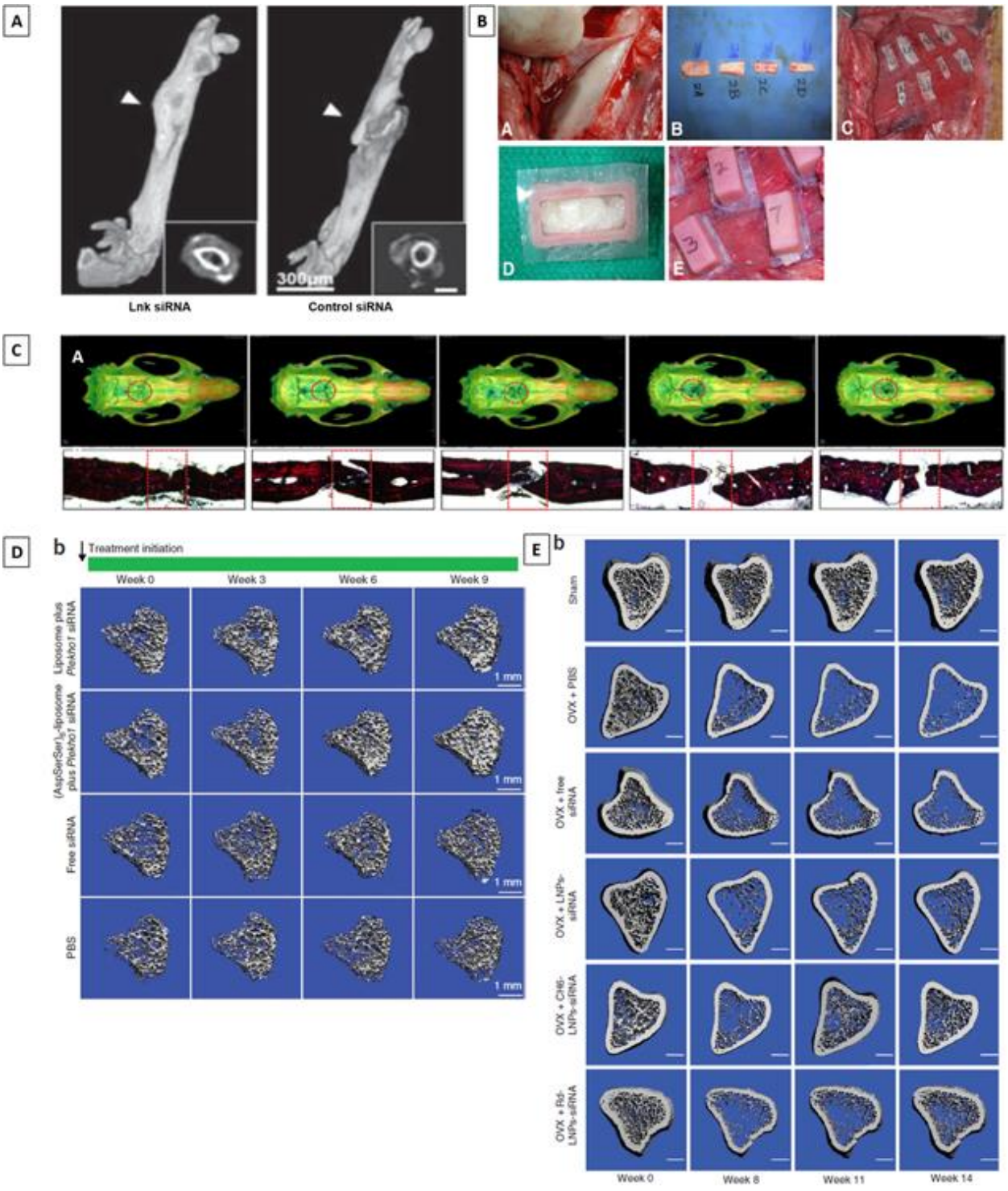


Figure 13. *(*A*) In vitro and in vivo, local delivery: μCT reconstruction images of previously fractured rat femurs, treated with Lnk siRNA or control siRNA (Kawakami, Ii et al. 2013). (*B*) In vitro and in vivo, local delivery: Representative immunohistological*

study of the intermolar regions in mice treated either with Sema4d siRNA or control: osteoblast and osteoclast numbers and Sema4d *gene expression and knockdown. Serial pictures demonstrating different steps of surgical procedure in sheep (Rios, Skoracki et al. 2012). (*C*) In vitro and in vivo, local delivery: Bone repair in the rat calvarial defect model, µCT imaging (top panel) and the corresponding histology imaging, Giessen's staining (bottom panel) (Jia, Yang et al. 2014). (*D*) In vitro and in vivo, systemic delivery: Serial in vivo µCT imaging of trabecular architecture of proximal tibias over the course of 9 weeks in animals treated with Plekho1 siRNA using different delivery methods, compared to control group (Zhang, Guo et al. 2012). (*E*) In vitro and in vivo, systemic delivery: Serial in vivo three-dimensional trabecular architecture of the proximal tibia over 14 weeks in mouse models of osteoporosis treated with different siRNA delivery methods (Liang, Guo et al. 2015).*

1.11.2. Lipid-based DDS

"Lipid-based DDS have been more widely used for transfecting cells with siRNA. As a matter of fact, almost all commercially available transfection reagents are modified cationic liposome-based systems (e.g. lipofectamine® from Invitrogen, DOTAP and DOSPER from Boehringer Mannheim Biochemicals, TransFast™ from Promega, Ambion® from Thermofisher, and SUB9 kits from Precisionnanosystems). Liposomes are core-shell vesicular structures with at least one lipid bilayer. For siRNA transfection applications, the hydrophilic heads of amphiphilic lipids used to fabricate liposomes are usually cationic in nature. Neutral lipids such as cholesterol are used to stabilize the lipid bilayer structure. Cationic liposomes can easily complex with anionic siRNA

through electrostatic attractions, and also adhere readily on the anionic membrane of cells. Moreover, liposomes have been commercially available for decades as DDS for various therapeutic agents (e.g. Ambisome), and therefore established methods are available for the upscale production of liposomes, which makes them attractive to pharmaceutical companies.

Commercially-available Ambion® (polyamine-based transfection agent) was used to transfect MSCs with siRNAs against guanine nucleotide-binding protein alpha-stimulating activity polypeptide 1 (GNAS1) and prolyl hydroxylase domain containing protein 2 (PHD2) to target core binding factor alpha 1 (Cbfa1) and hypoxia-inducible factor 1(HIF-1) pathways respectively (Rios, Skoracki et al. 2012). Activating GNAS1 leads to the proteolytic degradation of Cbfa1 (an osteogenic differentiation transcript factor), and therefore silencing GNAS1 can lead to enhanced osteogenic differentiation. On the other hand, silencing PHD2 promotes the expression of angiogenic factors through the hypoxia pathway. Although *in vitro* analyses were not decisive regarding the effectiveness of the GNAS1 and PHD2 siRNAs in increasing osteogenesis, *in vivo* results demonstrated some promising results. In a sheep model of bone formation, siRNA-loaded chitosan-silk fibroin scaffolds were implanted over the periosteum. By day 70 there was an increase in bone volume in scaffolds containing GNAS1, PHD2 and the combination of both siRNAs **Figure 13**B.

Lipofectamine 2000® is a widely used, highly cationic, lipid-based DDS utilized for siRNA delivery. Sen Jia et al. (Jia, Yang et al. 2014) investigated the effects of two different siRNA-Lipofectamine 2000 complexes targeting casein kinase 2interaction protein 1 (Ckip-1) and soluble VEGF receptor 1(siFlt-1) to promote both osteogenesis

and angiogenesis simultaneously on MSCs and also *in vivo*, using a skull critical size defect in rat models. These siRNA-Lipofectamine 2000 complexes were incorporated in a biodegradable lyophilized chitosan sponge as a scaffold for bone regeneration. The authors concluded that this scaffold system was able to maintain the siRNA efficiently over time and the synergistic effect of two different types of siRNA stimulated both osteogenesis and angiogenesis *in vitro* and enhanced bone regeneration *in vivo* **Figure 13**C. In a similar biological approach, Y. Xu et al. (Xu, Mirmalek-Sani et al. 2006) investigated the inhibitory effects of siRNA against PPAR-γ on suppression of adipogenic differentiation of cryopreserved human subcutaneous pre-adipocytes and also fresh fetal-femur derived MSCs. The authors used Lipofectamine 2000 for siRNA delivery to the cells and showed a consequent significant suppression of adipocyte differentiation capacity of this system *in vitro*. Lipofectamine 2000 complexed with siRNA was also used to knockdown the expression of Chordin (BMP inhibitor) in human MSCs (Kwong, Richardson et al. 2008). Chordin knockdown resulted in increased expression of osteoblastic cell marker (Alp) and extracellular mineral formation by these cells. Lipofectamine RNAiMAX is another liposome-based system that has been commercially provided for enhanced transfection of mesenchymal and neural stem cells. Elve Chen et al. studied the effects of silencing BRE, a multifunctional adaptor protein important in cell survival, DNA repair and stress response, on osteogenic and chondrogenic differentiation of human umbilical cord perivascular (HUCPV) multipotent progenitor cells (Chen, Tang et al. 2013). The authors found that the expression of BRE is decreased when chondrocytic or osteoblastic differentiation is induced in HUCPV mesenchymal cells. Therefore, Lipofectamine RNAiMAX was used to deliver siRNA

against BRE to HUCPV progenitors. Both osteogenesis and chondrogenesis were accelerated post- transfection. Ge Zhang et al. (Zhang, Guo et al. 2012) reported a systemically-administered and targeted approach to silence bone-formation-inhibitory genes utilizing siRNAs in osteogenic lineage cells. The group explored the use of DOTAP cationic liposomes targeting only bone-formation surfaces to deliver siRNA against Plekho1 gene in an *in vivo* osteoporotic rat model. The gene Plekho1 was discovered recently as a negative regulator of osteogenic lineage activity with little effect on bone resorption. The proposed cationic liposome in this study had a high binding affinity to lowly crystalized hydroxyapatite due to an (AspSerSer) 6 moiety. As such, this strategy was found effective in targeting specifically the bone forming surfaces of the skeletal system and delivering the cargo to the osteogenic-lineage cells **Figure 13**D. In a recent paper, the group reported on more efficient application of the same siRNA through using an osteoblast-specific aptamer surface-functionalized lipid nanoparticle to deliver Plekho1 siRNA specifically to bone. Systemic application of this siRNA resulted in Plekho1 gene silencing followed by improved bone formation and bone microarchitecture and increased mechanical properties, tested in both healthy and osteoporotic animal models **Figure 13**E (Liang, Guo et al. 2015).

Lipidoids - another class of lipid-based nanoparticles - have a similar structure to liposomes and are synthesized by conjugating alkyl-acrylamides and amine molecules. They require fewer steps for fabrication compared to liposomes, which makes them preferential for high throughout analysis of different therapeutic agents (Fulmer 2010). Moreover, it has been shown that at a low serum level (2%), the transfection of HUVEC endothelial cells using lipidoid was comparable to Lipofectamine

2000. However, at a high serum level (10%) lipidoids had superior transfection efficiency compared to Lipofectaime 2000. This is another advantage of using lipidoids over cationic liposomes, which easily aggregate in the presence of high serum concentrations. Ramasubramanian et al (Ramasubramanian, Jeeawoody et al. 2015) examined the effects of delivering BMP-2 DNA in conjunction with siRNA against both Noggin and GNAS to a human fetal osteoblast (immortalized) cell line using a lipidoid called NA114, which was previously demonstrated to be an efficient nanoparticle for siRNA delivery (Cho, Goldberg et al. 2009) cell transfection with BMP-2 DNA or Noggin and GNAS siRNA resulted in increased cell apoptosis, decreased proliferation and viability compared to sham DNA or siRNA treated cells. The authors concluded that the response of progenitor cells and immortalized cell populations to exogenous osteogenic gene delivery is different and highlighted the need for a targeted gene delivery mechanism for bone regeneration.

1.11.3. Other DDS

Ahmed El-Fiqi et al (El-Fiqi, Kim et al. 2012) fabricated and utilized bioactive glass nanoparticles (BGn) smaller than 100 nm with mesopores sized 3-5 nm for delivery of both small chemical drugs (Na-ampicilin) and also small nucleic acids (siRNA). They showed a sustained release profile of siRNA from such nanoparticles up to three days and a high cell transfection efficiency (roughly 80%) followed by about 85% target gene silencing in HELA cells. The authors examined the cytotoxicity of their delivery system on mouse pre-osteoblastic cell line and rat bone marrow stromal cells.

A summary of preclinical studies employing siRNA approaches directly for bone regeneration is provided in

Table 3.”

Table 3. Summary of Preclinical Investigations Employing siRNA Approaches to Study Bone Regeneration

***In vitro* only**					
Ref #	**Gene(s) of Interest**	**Delivery method**	**Delivery Reagent**	**Cell type**	**Outcome summary**
(Hong, Wei et al. 2012)	glucocorticoid receptor (GR)	Polymer microparticle	PLGA	human bone marrow	Successful siRNA delivery and release for up to 40 days, successful *in vitro* transfection
(Wang, Tran et al. 2012)	RANK	polymer	PLGA	Murine osteoclast precursor cells	Targeted phagocytic cell-specific siRNA delivery, inhibition of bone resorption due to RANK expression knock-down in osteoclasts and their precursor phagocytic cells
(Nguyen, Jeon et al. 2014)	GFP, Luciferase, Noggin	polymer	branched PEI	Human MSC	Enhanced osteogenic differentiation of hMSCs due to prolonged (7 weeks) bioactivity of Noggin siRNA
(Xu, Mirmalek-Sani et al. 2006)	PPARγ	Lipid	Lipofectamine 2000™	human subcutaneous preadipocytes, Human fetal-femur-derived MSCs	PPARγ-siRNA is a useful tool to study adipogenesis in human cells, with potential applications both therapeutic and in the elucidation of mesenchymal cell differentiation in the modulation of cell differentiation in human mesenchymal cells.
(Kwong, Richardson et al. 2008)	Chordin	Lipid	LIpofectamine 2000™	Human bone marrow MSCs	Significant increase in the expression of alkaline phosphatase and extracellular mineral deposition as a result of *Chordin* knock down by siRNA
(Chen, Tang et al. 2013)	BRE	Lipid	Lipofectamine™ RNAiMAX	HUCPV progenitor cells	Silencing BRE promoted stemness and inhibited the differentiation of HUCPV cells
(Ramasubramanian, Jeeawoody et al. 2015)	GNAS, Noggin	Lipid	Lipidoid NA114C	human fetal osteoblasts	decreased cell viability in a dose-dependent manner and a general increase in cell apoptosis
(El-Fiqi, Kim et al. 2012)	bcl-2	Inorganic bioactive nanomaterials	Bioactive glass nanoparticles (BGn)	HeLa cell line, preosteoblastic MC3T3-E1 cells, and rat bone marrow mesenchymal stem cells	Satisfactory siRNA loading, release and cell transfection
In vivo only					
Ref #	Gene(s) of Interest	**Delivery method**	**Delivery Reagent**	**Animal Model**	**Outcome summary**
(Zhang, Wei et al. 2014)	Semaphorin 4d	polymeric nanoparticles	Asp8-(STR-R8)	Ovariectomy in mice	decreased bone loss resulted from osteoporosis
In vitro and In vivo					
Local Delivery					

Ref #	Gene(s) of Interest	Delivery method	Delivery Reagent	In vitro (Cell type)	In vivo (Animal Model)	Outcome summary
(Kawakami, Ii et al. 2013)	Lnk	Lipid	Lipofectamine TM	Mouse bone marrow and osteoblast cells	Transverse femoral shaft Fracture in mice	In vitro: Lnk siRNA-transfected osteoblasts showed highly osteoblastic capacity In vivo: Enhanced fracture repair in the Lnk-siRNA treated group
(Rios, Skoracki et al. 2012)	GNAS1, PHD2	biomaterial	silk fibroin-chitosan (SFCS)	Human mesenchymal stem cells	Autograft, sheep	Both siPHD2 and siGNAS1 supported bone regeneration in vivo, only siGNAS1 regulated bone phenotype in vitro
(Jia, Yang et al. 2014)	siCkip-1, siFlt-1	Lipid	Lipofectamine 2000 TM	Rat bone marrow MSCs	Skull critical size defect, rat	Significant suppression of target genes, new bone formation in vivo
Systemic Delivery						
(Zhang, Guo et al. 2012)	Plekho1	Lipid	(DOTAP)-based cationic liposomes with six repetitive sequences of aspartate, serine, serine ((AspSerSer)6)	human osteoblast-like cells (hFOB 1.19 cells) , human osteoclast-like cells (giant-cell tumors)	systemic delivery in rats	Systemic delivery of Plekho1 siRNA in rats resulted in the selective enrichment of the siRNAs in osteogenic cells and the subsequent depletion of Plekho1
(Liang, Guo et al. 2015)	Plekho1	Lipid	aptamer–functionalized lipid nanoparticles (LNPs)	rat primary osteoblasts, rat liver cell line BRL-3A and rat PBMCs, human primary osteoblasts, human osteoclasts, human liver cells (THLE-3)	systemic delivery in rats	In vitro osteoblast-selective uptake of Plekho1 siRNA, in vivo osteoblast-specific Plekho1 gene silencing, which promoted bone formation, improved bone microarchitecture, increased bone mass and enhanced mechanical properties in both osteopenic and healthy rodents.

In the current study, Noggin siRNA has been used to inhibit Noggin signaling in rat originated UMR osteoblastic cells. Lipid nanoparticles were employed for cell delivery of this siRNA. As it will be described in detail in the following chapters, initially a lipid core layer by layer (Alginate- Chitosan) nanoparticle was designed, fabricated and characterized for siRNA delivery. According to the encapsulation and delivery efficiency of the above nanoparticles, we tested a second lipid nanoparticle for the same purpose. Lipofectamine 2000 was employed to experiment the effects of Noggin inhibition on osteoblastogenesis and mineralized matrix formation of UMR cells in culture and a few 3D culture and *in vivo* experiments as proof of concept.

2. Materials and Methods

2.1. Study Design

The *in vitro* basic experimental part of the study was designed to address our hypothesis: the delivery of Noggin siRNA from LNPs would be efficient and can lead to enhanced osteoblastic differentiation and bone formation compared to control siRNA.

All experimental procedures complied with national and institutional guidelines at University of McGill. Our primary outcome was to investigate whether Noggin inhibition in pre-osteoblasts could result in enhanced osteoblastic differentiation and mineralized matrix formation. Rat osteoblastic cells derived from a sole source were utilized for the experiments. Independent variables consisted of the cultured cells and their medium containing different concentrations of hBMP-2, lipid nanoparticles and Noggin siRNA. The dependent variables included the Noggin and other osteoblast-specific gene expression, Noggin protein expression, Alp activity, cell death and mineralized matrix production by osteoblasts. All the cell types and reagents that were utilized in the experiments are listed below.

2.2. Formulation of Liposomes

The liposomes were made by the thin-film hydration technique, using the protocols verified in the lab. A lipid solution was made by dissolving 100 mg 1, 2-Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC: Genzyme Pharmaceuticals, Switzerland), 26.3 mg cholesterol (Sigma-Aldrich Chemical) and 5.15 mg of a cationic surfactant; dimethyldioctadecyl-ammonium bromide (DDAB: Sigma-Aldrich Chemical) in 5 ml chloroform-methanol (Fisher Scientific) mixture (4:1, v/v). 4% molar DDAB was used to modify the surface charge of the liposomes. The solvent was then evaporated from the lipid phase using a rotary evaporation device under 50 cmHg vacuum. This

yielded a dry homogenous lipid film. This film was hydrated using 5 ml of double distilled water, and then vortexed at high speed for 10 minutes resulting in a suspension of multi-sized cationic multi-lamellar liposomes. The solution was extruded using a mini extruder (Avanti® Polar Lipids, Inc.) with polycarbonate filters (GE Osmonics) at two different pore sizes of 200 nm and 100 nm.

2.3. Preparation of layer by layer (LBL) Lipid Nanoparticles

Two different solutions of alginate (AL) and chitosan (CH) 1mg/mL were prepared in PBS (Sigma-Aldrich Chemical). Alginic acid (low viscosity; 12 kDa molecular weight) and chitosan (85% deacetylated; 91.11 kDa molecular weight) (both from Sigma-Aldrich Chemical) were used to make LBL liposomes. To make LBL cationic liposomes, they were poured into alternating AL and CH solutions so layers of negatively charged AL and positively charged CH were built up. After each cycle of layering the liposomes, they were centrifuged at 1600*g* for 15 minutes in order to remove the excess AL and CH in the solution that was not attached to the surface of the liposomes.

2.4. Characterization of LBL Nanoparticles

2.4.1. Average particle size, surface charge and physical stability

Average hydrodynamic diameter (size), size distribution (polydispersity index; PI) and surface charge [zeta (ζ) potential] was measured by ZetaPALS instrument with a particle sizing option (Brookhaven Instruments, USA). Sucrose was used as a

cryoprotectant prior to freeze drying the samples at -54ºC for 48 hours (Thermo Savant, Modulyo D-115).

2.4.2. Oligonucleotide Encapsulation Efficiency and Loading Capacity

The lyophilized nanoparticles were weighed and then re-hydrated using PBS with or without DNA/siRNA to the original volume relative to their initial 5ml volume. The DNA/siRNA-loaded particles were separated from the un-adsorbed oligonucleotides by ultracentrifugation for 30 minutes at 180000*g* and 25°C (Beckman TL-100 Ultracentrifuge). Un-adsorbed DNA/siRNA with a fluorescent tag in the supernatant and the samples was quantified using spectrophotometry by reading the absorbance at 570 nm using SpectraMax i3 plate reader (Molecular Devices, CA, USA).

2.4.3. Oligonucleotide Retention Study

Aliquots of nanoparticle suspensions loaded with DNA/siRNA with florescent tag were maintained at 37°C. Suspensions were then ultracentrifuged for 20 minutes at 180000*g* and 25°C to separate the nanoparticles from the supernatant containing released oligonucleotides for quantitative analysis. The pellet was re-suspended in 3 ml of fresh PBS. The amount of released oligonucleotide was analyzed spectrophotometrically by measuring the florescent signal intensity both in the supernatant and in the resuspended liposome mix using SpectraMax i3 plate reader (Molecular Devices, CA, USA), reading the absorbance at 570 nm.

Additionally, we checked the particle size change during the above process.

2.4.4. Surface Charge and Physical Stability

During the sequential adsorption of alginate and chitosan we also measuring the ζ-potential upon addition of each polyelectrolyte layer, before and after the centrifugation of the particles. In addition to providing the evidence for the presence and coverage of the polymer coating (Alginate and then Chitosan), ζ-potential is a significant indicator of the stability of nanoparticulate suspensions. ζ-potentials above +30 mV or below -30 mV are commonly considered to be an indication of stability and greater uniformity by inducing strong repulsion forces between particles which prevent aggregation, too (Gallardo, Morales et al. 2005) .

We freeze-dried the liposome batch after the layers application. The rationale behind this was two-fold: (a) additional stability in terms of particle size and surface charge prior and after lyophilization; and (b) rehydrating the lyophilized nanoparticles with siRNA/DNA dissolved in PBS to evaluate their loading capacity, encapsulation efficiency and release profile. We anticipated that as the lyophilized nanoparticles are being rehydrated, they would allow the oligonucleotides inside the liposome core, rather than surface adsorption.

We used sucrose to prevent particle fusion or aggregation as it has been stated in the literature that it is able to do so by acting as a spacer between nanoparticles (Womersley, Uster et al. 1986).

2.5. Cell Culture

Rat osteosarcoma cell line, UMR-106 cells, (donation from Dr. Pierre Moffatte) were maintained in Dulbecco's modified Eagle's medium (DMEM, Life Technologies®, ON, Canada) at 37°C with 5% CO2, supplemented with 10% heat inactivated fetal calf

serum (FBS, Life Technologies®, ON, Canada) and 1% antibiotics (Penicillin/Streptomycin). For hBMP-2 (R&D Systems®, ON, Canada) treatment or siRNA transfection, cells were initially plated at $4x10^4$ /well in 24 well plates and after 24 hours, culture medium was removed and cells were rinsed using 1x phosphate buffer saline (PBS) and treatment and/or transfection were carried out as described below. Quantification of cell transfection efficiency by fluorescence activated cell sorting (FACS), measurement of alkaline phosphatase activity, and Alizarin Red Assay were carried out at indicated time points. For osteoblastic differentiation experiments, the growth medium was supplemented with 10 mM β-glycerophosphate and 250 µM ascorbic acid (both from Sigma-Aldrich, Ontario, Canada)) and the culture was continued for an additional period of 10 days. The culture medium was changed every third day **Figure 14**.

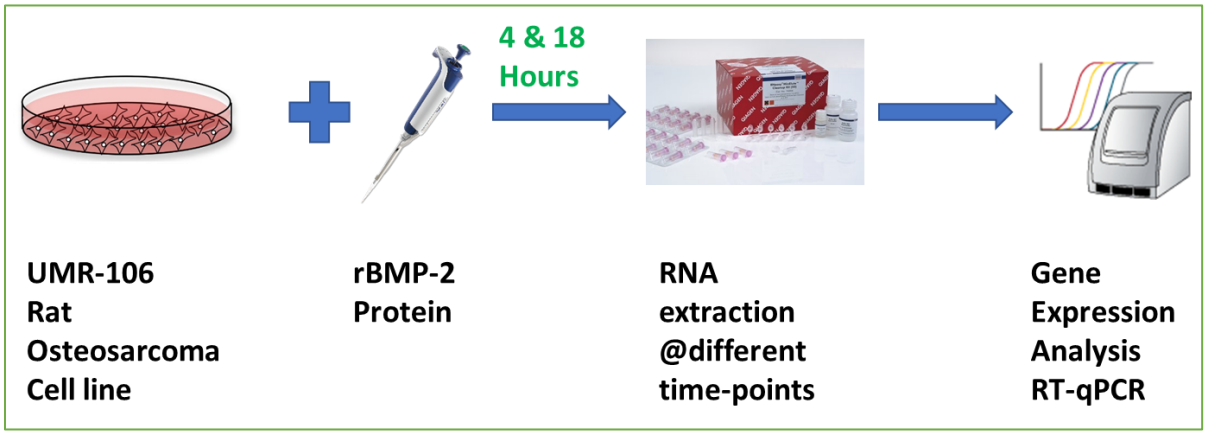


Figure 14. *Schematic steps for gene expression analysis from cultured UMR-106 cells.*

2.6. Alkaline phosphatase activity

Alkaline phosphatase (ALP) activity was determined in cell lysates using a colorimetric Alkaline Phosphatase Assay kit (Abcam Inc., Toronto, ON) according to the manufacturer's protocol. ALP enzyme activity was normalized to the total protein content of the cell lysates from corresponding samples, measured by a Pierce BCA Assay kit (Thermo Fisher Scientific®, Ottawa, Ontario).

2.7. Gene expression

Gene expression was examined by quantitative real-time PCR. Total RNA from D1 cell cultures was isolated using the RNeasy Plus Mini Kit (Qiagen Inc., Ontario) as described in the manufacturer's protocol. Total RNA was treated with DNase and reverse transcribed using the Maxima First Strand cDNA synthesis kit with ds DNase (Thermo Fisher Scientific®, Ottawa, Ontario). Before use, RT samples were diluted 1:5. Gene expression was determined using assays designed with the Universal Probe Library from Roche (www.universalprobelibrary.com). For each qPCR assay, a standard curve was performed to ensure that the efficiency of the assay is between 90% and 110%. qPCR reactions were performed using PERFECTA QPCR FASTMIX II (Quanta), 2 µM of each primer and 1 µM of the corresponding UPL probe. The Viia7 qPCR instrument (Life Technologies) was used to detect the amplification level and was programmed with an initial step of 20 second at 95˚C, followed by 40 cycles of: 1 second at 95˚C and 20 second at 60˚C. Relative expression (RQ = 2-△△CT) was calculated using the Expression Suite software (Life Technologies), and normalization was done against Actb.

The probe/primer combinations that were used in our qPCR experiments are listed in **Table 4**.

Table 4. List of primers used in RT-qPCR and the reference IDs of corresponding genes.

Gene	Reference ID	Forward Sequence	Reverse Sequence
Actin	NM_031144	cccgcgagtacaaccttct	cgtcatccatggcgaact
Noggin	NM_012990.1	cctgagcaagaagctgagga	accgggcagaaggtctgt
Smad1	NM_013130.2	ccactataagcgagtggagagc	aggctgtgctgagggttgta
Smad5	NM_021692.1	ataacaagagccgcttctgc	ccaccaacgtagtatagatggaca
Runx2	NM_053470.2	catccatccattccaccac	ggtggcagtgtcatcatctg
Ibsp	NM_012587.2	gcgatagttcggaggagga	cccctcagagtcttcgttgt
Gremlin	NM_019282.2	aggatccactgaggtgacaga	cagctgctggcagtaggg
Chordin	NM_057134.1	gaaccagcgcactgtcct	tcattctgtagcagcatgtgag

2.8. Cell toxicity assay

Cell toxicity was evaluated using a LIVE/DEAD® Viability/Cytotoxicity Kit, for mammalian cells according to the manufacturer's instructions. Briefly, 1x105 UMR cells were cultured in 12 well plates for 24 h. The cells were then washed with 1x PBS thoroughly and were treated with Lipofectamine 2000 (Thermo Fisher Scientific®, Ottawa, Ontario), negative control siRNA or the combination of Lipofectamine 2000 and the siRNA. Treatment groups were all in triplicate. The following day, 300 ul of live/dead assay reagent was added to each well in a group of treatment at a time. The samples

were then incubated at 37℃ in the staining solution for an additional 15 min followed by immediate fluorescence microscopy imaging. Images were taken from six different microscope fields randomly. ImageJ software was used to count the total number of live and dead cells in each field of the treatment groups and the final number of live or dead cells was calculated by averaging the six obtained numbers from all fields. The viability percentage was calculated according to the following equation:

$$\textbf{Viability } \% = \frac{\textbf{Number of Live cells}}{\textbf{Number of total cells}} \textbf{ x 100 } \%$$

2.9. Protein expression (ELISA)

Expression of Noggin protein in cultured cells was measured using a sandwich enzyme immunoassay ELISA kit (Cloud- Clone Corp.) according to the producer's protocol. In summary, 100µL standard or sample was added to each measurement well and the samples were incubated for two hours at 37℃. 100µL of prepared detection reagent A was added to each sample and incubated for one hour at 37 ℃. The samples were washed three times before 100µL prepared detection reagent B was added. After 30 minutes incubation at 37℃ the samples were washed 5 times and 90µL of substrate solution was added and incubated for 20 minutes at 37℃. Finally, 50µL of stop solution was added and the plate was read at 450nm using a SpectraMax i3 plate reader

(Molecular Devices, CA, USA). **Figure 15** All the ELISA experiments were performed in triplicate.

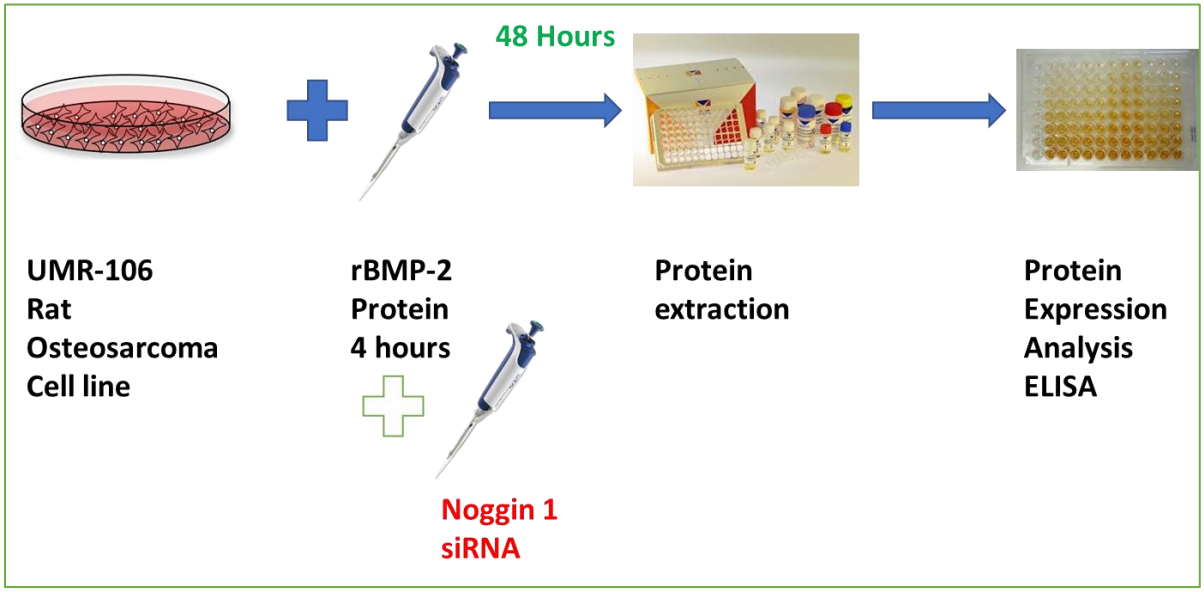


Figure 15. *schematic steps for protein expression analysis from UMR-106 cells treated with rBMP-2*

2.10. Noggin siRNA screening

siRNA-mediated Noggin down-regulation was investigated in UMR-106 rat pre-osteoblasts. Each sample was evaluated for Noggin expression normalized to Actin in triplicate using qPCR as explained above. The Noggin siRNA inducing the most significant Noggin suppression was evaluated for protein suppression in UMR-106 rat pre-osteoblasts by ELISA assay and was employed in the subsequent osteoblastic differentiation experiments **Figure 16**.

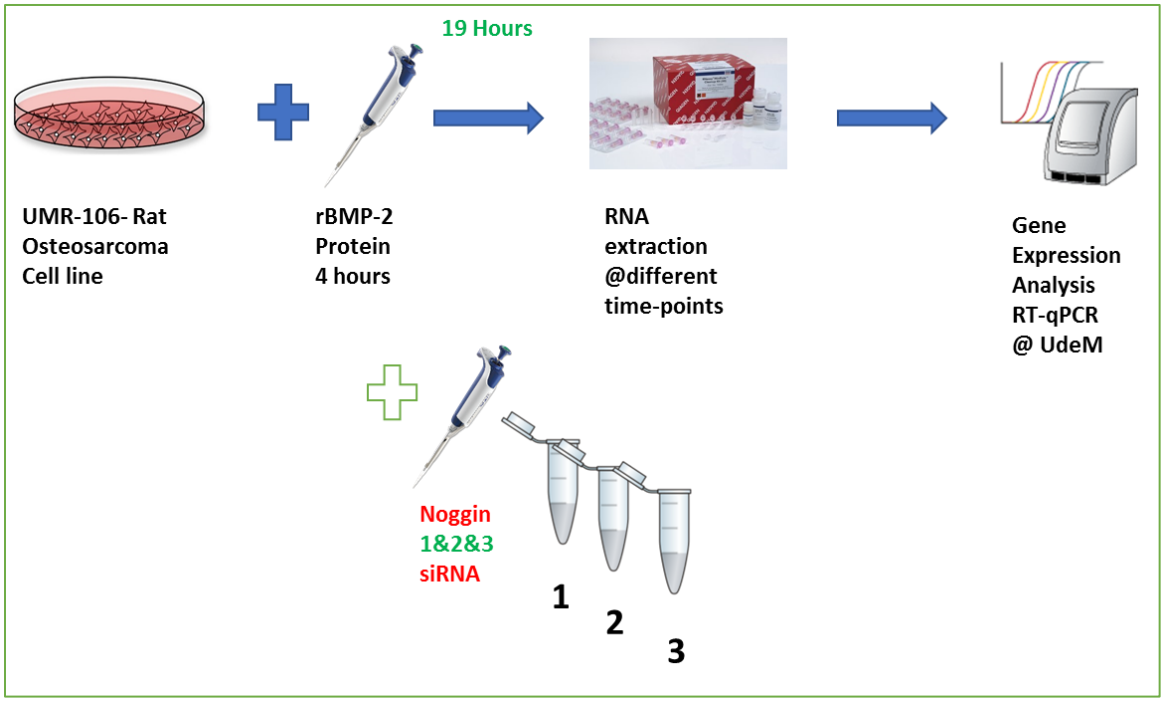


Figure 16. *Schematic steps of Noggin siRNA treatment after rhBMP-2 treatment and gene analysis to screen for the most efficient siRNA against Noggin.*

2.11. Histological analysis (Alizarin Red Assay-staining and quantification)

After 10 days of the osteoblastic differentiation culture, UMR cells were stained for calcium deposition by alizarin red S (ARS) staining (Sigma-Aldrich). UMR cells were fixed in cold 4% paraformaldehyde for 5 minutes, washed three times with 1x PBS and stained with 1% ARS (pH adjusted to 4.3) for 15 minutes at room temperature. At this point, the ARS was removed from the plates by gentle suction and the cells were washed by 1x PBS until the PBS was clear. The stained cells were observed by microscopy and images were captured from each well using a Canon EOS 60D camera

(Canon). **Figure 17** The stained cells were de-stained with 10% acetic acid (Sigma-Aldrich). To each well of a 24 well plate, 200 µl acetic acid was added and incubated at room temperature for 30 minutes with moderate shaking. A 100 µl aliquot of the eluted dye from each well was transferred to a well of a 96-well plate and the absorbance was measured at 562 nm using a SpectraMax i3 plate reader (Molecular Devices, CA, USA).

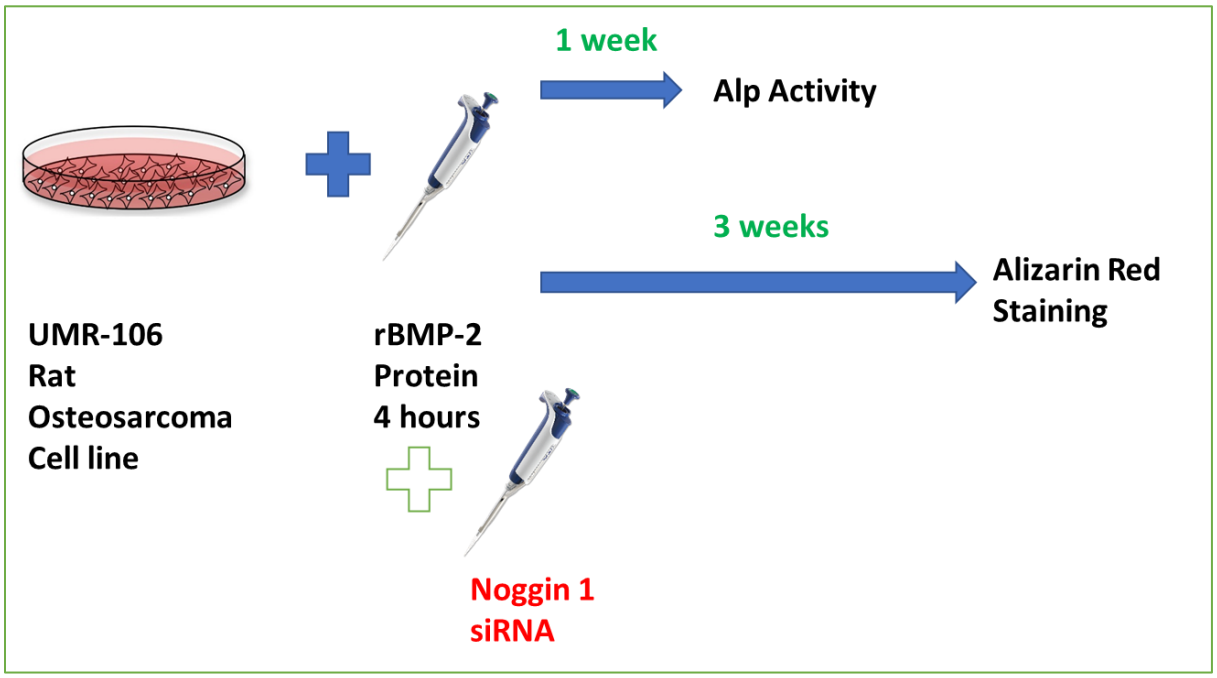


Figure 17. *Schematic steps of Alp activity analysis and Alizarin Red staining*

2.12. Collagen scaffold trial

To study the properties of a collagen hydrogel/scaffold for noggin-siRNA delivery to bone defects, we prepared Collagen Type 1 scaffolds from rat tail Col-1.

For the purpose of this experiment, the mixture of Lipofectamine-siRNA was prepared according to the protocols previously explained here and we used on cultured cells. However, instead of adding the siRNA-lipid cocktail to the cultured cells, we first

detached the cells and rinsed them with PBS, the cells were then resuspended in normal growing medium (DMEM) with 10% FBS and the volume of this mixture was topped with extra medium. The collagen gel was diluted with varying volumes of the final solution containing all the transfection reagents to optimize the methodology.

Different experimental groups had varying amounts of Col 1 gel and cell mixture:

Group 1: 30% Col 1 Gel, 70% Cells+ siRNA+ Lipofectamine in Medium

Group 2: 50% Col 1 Gel, 50% Cells+ siRNA+ Lipofectamine in Medium

Group 3: 75% Col 1 Gel, 25% Cells+ siRNA+ Lipofectamine in Medium

Group 4: 100% Col 1 Gel

Group 5: 50% Col 1 Gel, 50% Lipofectamine in Medium

The gels were kept in sterile tissue culture hood 37 C and 5% CO2 for either 24 hours or 10 days of culture and the events were monitored during the culture time (to check for contamination, shrinkage, etc.) until the endpoint.

Following each timepoint, the samples were fixed using 10 % formaldehyde and prepared for electron microscopy analysis in a collaborator's lab.

2.13. Animal trial

In order to experiment the feasibility of the proposed *in vitro* methodology in an animal model and to investigate the translational possibility of Noggin inhibition approach for future CSD and fracture repair, an animal trial was designed and proof of

concept experiment was carried out on C57Black/N6 wild type mouse tibial fracture model at 8-12 week of age.

Mice were acquired, received and acclimatized and then underwent rodded fracture surgery as per approved SOPs at McGill and Shriners Hospital for Children (Supplementary document).

Briefly, for open tibial fracture surgery, the mice are first anesthetized with Isoflurane. The hair around the right hind limb is completely shaved. The skin is sterilized and then a small incision is made on the skin, above the pattella to visualize the tibial plateau, an entry point is made using 26 G needle into the medullary canal of the tibia. Through the 26 G needle, the internal wire guide of a 25G BD Spinal Needle is inserted into the canal. Then the 26 G needle is pulled up and used to bend the spinal needle to 90 degrees. Using the tips of delicate tweezers, an incision below the tibia around the midshaft area is created, and then the tibia is gently cut using a surgical fine scissors. At the area of this induced fracture, 10 ul mixture of siRNA-Lipid mixed with Collagen at 50:50 volume is injected. The wound is sutured and the animals are allowed unrestricted weight bearing.

Imaging analysis of the fractures consisted of X-ray (Faxitron) and *In vivo* Extreme. Optical imaging procedures:

Animals were transported to the SAIL Imaging Suite at RIMUHC, McGill. Animals were anesthetized by 4-5% isoflurane. While anesthetized, the animal was moved to the Bruker In-Vivo Xtreme optical imager and placed in the supine position in an imaging tray. The animal was maintained at ~37°C using an air-warming system. Following

completion of imaging, the animal was given approximately 0.5 mL of sterile, warmed saline subcutaneously, returned to the procedure suite, and monitored during recovery from anesthesia under a warming lamp.

2.14. Statistical Analysis

Statistical significance was assessed using student t-test and one-way analysis of variance, followed by Tukey multiple comparisons post-test, where applicable. The GraphPad software 5.0 and statistical package (Prism®) was used to perform the statistical analysis and to generate the graphs.

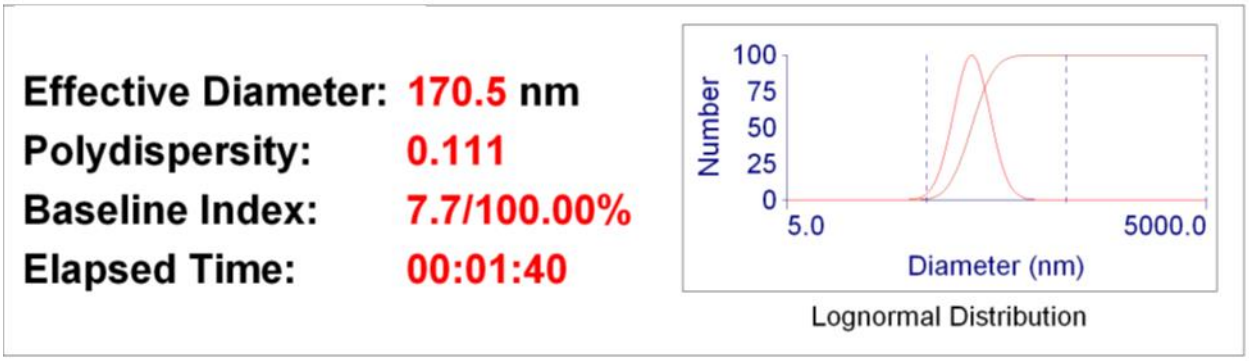
3. Results

3.1. Liposomes: Fabrication, characterization and encapsulation efficiency

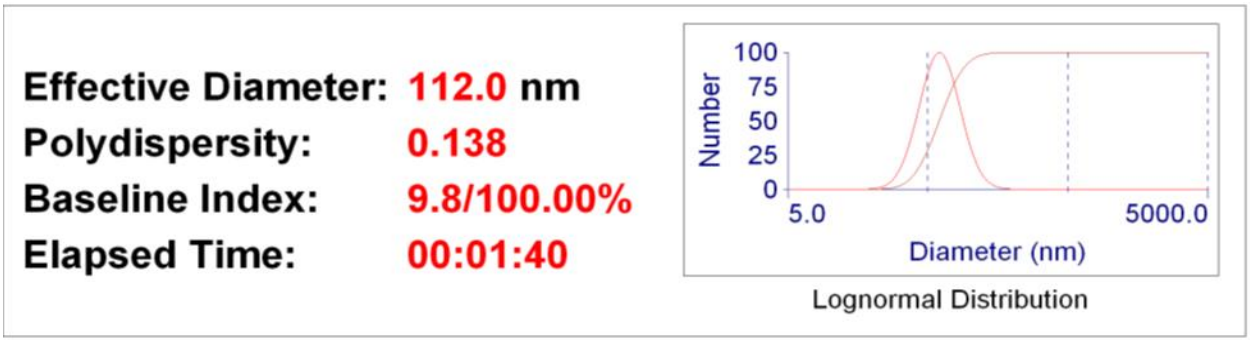
According to the established protocols in the lab and as described in the methods, liposomes were fabricated (200 nm) and lyophilized to keep a stock for future applications.

As shown below, we checked the size of nanoparticles at various stages of liposome preparation to ensure the correct size throughout the experiment. **Figure 18 A**. shows the size of liposomes after extruding them through a 200nm filter. We had liposomes with an average size of 170 nm. **Figure 18 B**. Shows the size of the same liposomes after lyophilisation and hydration with PBS; and **Figure 18 C**. Shows their size after filtration/centrifugation to discard excess PBS. This data shows that the size of liposomes becomes slightly smaller after hydration and remains unchanged after centrifugation. Also, the polydispersity values (roughly 0.1) show that almost no aggregation of the liposomes occurred during various stages of this process.

A



B



C

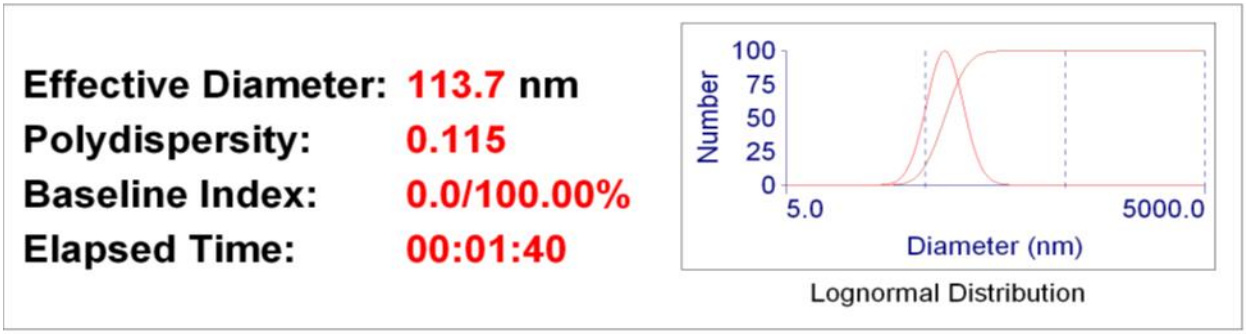


Figure 18. *Diameter, Zeta potential and polydispersity index measured for liposomes. A. After preparation and extruding B. After rehydration of lyophilised liposomes C. After centrifugation of rehydrated liposomes*

A trial experiment was carried out for oligonucleotide encapsulation in the liposomes. A mix of oligonucleotide with PBS was prepared to re-hydrate the lyophilized liposomes to a final concentration of 100nM in 5ml PBS. After incubation at room

temperature, the samples were centrifuged using 50KDa filter columns to separate the excess oligonucleotide. Samples were then examined by fluorescent microscope to estimate the encapsulation according to the amount of fluorescent (data not shown). This experiment confirmed the encapsulation of oligonucleotide in the liposomes, although the signal was not very strong. Measurement of the fluorescent intensity by spectrophotometry at 570 nm also confirmed the encapsulation of fluorescent oligonucleotide into the liposomes (Data not shown).

3.2. Layer by layer liposomes

Layer by layer (LBL) liposome fabrication was carried out by applying two layers of chitosan and alginate on the liposomes according to the protocols available in the lab. As we faced some issues with the aggregation of liposome, we tried to optimize the protocol to minimize the aggregation in the process.

A fresh batch of 200nm liposomes was fabricated and two layers of alginate and chitosan were applied on the liposomes. This process was repeated twice to assure the success of the experiment and for troubleshooting **Figure 19**.

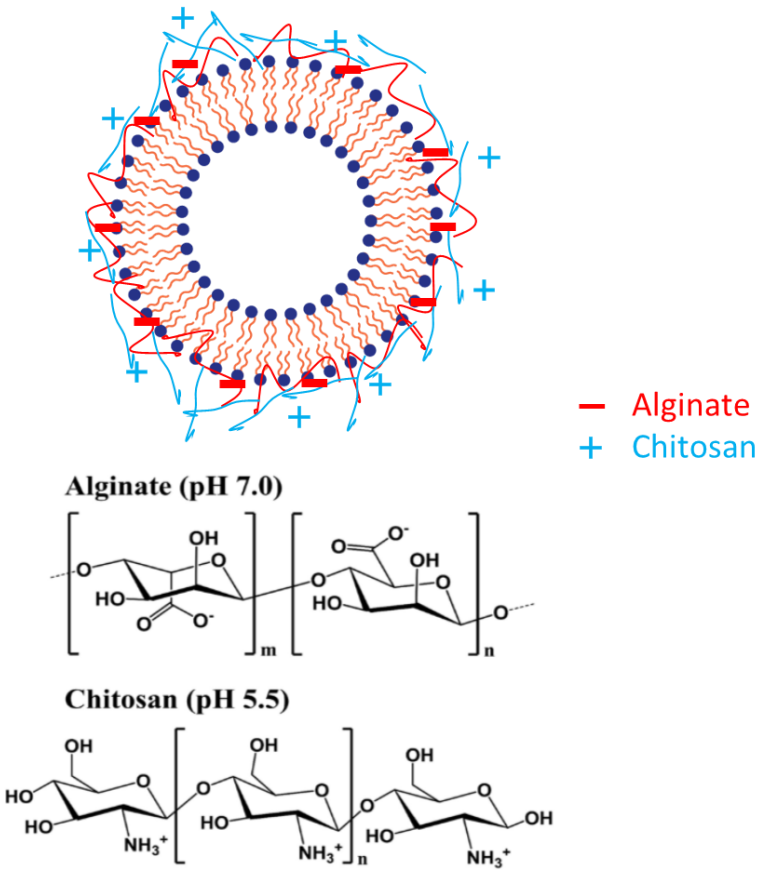


Figure 19. *Schematic figure demonstrating the LBL cationic liposomes, first covered by negatively charged Alginate followed by another layer of positively charged Chitosan.*

The mean size of initial liposomes was 145 ± 10 nm versus 355 ± 10 nm for liposomes coated with two polyelectrolyte layers. It is important that the adsorption of the chitosan layer on the previously adsorbed alginate layer seems to result in a decrease in size. This phenomenon could be explained by the ability of the shorter polymer chains of alginate to penetrate the layer of chitosan as a result of the strong ionic electrostatic interactions between these two polymers, which forms a denser network (Calvo, Remunan-Lopez et al. 1997). Furthermore, the polydispersity is indicative of particle consistency, ranging from 0 to 1. Values between 0 and 0.3 are

considered to represent a relatively homogeneous dispersion solution (Al Kobiasi, Chua et al. 2012).

Furthermore, we proceeded with freeze drying the LBL liposomes and then rehydrated them with PBS, containing fluorescent-labelled oligonucleotide to observe the encapsulation properties of these liposomes. Following centrifugation and PBS wash cycles, the fluorescent signal intensity in aliquots from each group and from the supernatants was quantified using spectrophotometry by reading the absorbance at 570 nm using SpectraMax i3 plate reader (Molecular Devices, CA, USA). The following figure demonstrates the comparison between the encapsulation of oligonucleotide in different LBL liposome groups (**Figure 20**).

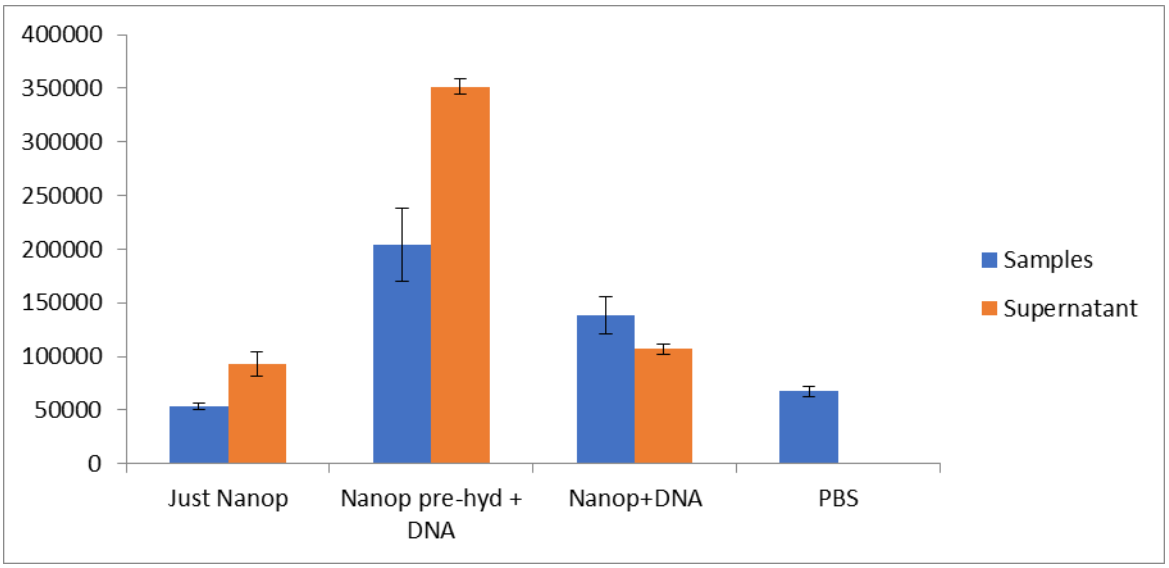


Figure 20. *Encapsulation of fluorescently tagged DNA in the LBL liposomes.*

The limitation of the above technique and protocol is the very low concentration of the LBL liposomes yielded. To overcome this limitation, we planned to prepare larger

volumes of highly diluted LBL liposomes and then increase the concentration to achieve massive quantities of LBL liposomes. Then we could lyophilize the liposomes under proper conditions and prepare sufficient dried LBL liposomes which then could be rehydrated with the PBS containing control siRNA (with fluorescent tag) for *in vitro* applications.

We next tried to compare the LBL liposomes made in the lab with another commercially available lipid nanoparticle, Lipofectamine 2000. We compared the particle size, Zeta Potential, chemistry of components (as much as possible to acquire information from the company's patent documents and other relevant publications found in the literature), the siRNA encapsulation efficiency, effect of high speed centrifugation on the retention of encapsulated siRNA (a necessary step for the purification of the inhouse made liposomes), and finally the cell transfection efficiency (on cultured UMR cells) of the nanoparticles.

The following **Table *5.*** The comparison of LBL nanoparticle fabricated in the lab with Lipofectamine 2000. summarizes the results of this comparison:

Table 5. *The comparison of LBL nanoparticle fabricated in the lab with Lipofectamine 2000.*

	LBL	**Lipofectamine**
Zeta potential	+30	> +90
Size (approx.)	200 nm	300 nm
Contains Sugar?	Yes	Most likely No

Encapsulation efficiency	<40%	>85%
Retention of siRNA after centrifuge x 2 times	30-40%	90-100%
Cell transfection efficiency (based on fluorescent + cells)	Low (<10%)	High (>80%)
Apparent cell toxicity	No	No

In summary, Lipofectamine appeared to be extremely cationic, slightly larger with enormous encapsulation efficiency. Following two cycles of high speed centrifugation, almost all the siRNA remained attached to the liposomes confirmed by tiny amounts of fluorescent detected in the supernatant.

In an effort to compare cell transfection properties of our LBL nanoparticle with Lipofectamine, less than 10% of cell transfection was achieved using LBL nanoparticle, compared to over 80% in Lipofectamine group. However, no apparent cell toxicity leading to cell death in the first 48 hours was detected in either groups.

The low efficiency of LBL LNPs in siRNA cell transfection was surprising as similar LNPs proved to be very successful in delivering BMP 7 to osteoblasts. However, it is important to note that in order for BMPs to act on cells, they need to bind their cell surface receptors, in another word, although LBL LNPs could not successfully cross the

cell membrane to deliver siRNA in the cytoplasm, they successfully delivered their cargo in the extra cellular matrix to interact with their receptors.

We concluded that the design of our LBL system, could not be changed drastically to reach a comparable level of success in siRNA delivery like Lipofectamine. Based on discussions with the supervisors, the plan was to continue the rest of the project, utilizing Lipofectamine as the LNP for siRNA delivery.

3.3. Noggin expression in response to rBMP-2 treatment

The Noggin negative feedback loop in response to rBMP-2 treatment at different time points using qPCR is shown in Figure 1. Treatment with rBMP-2 (100 ng/ml) led to a 5-fold and 9-fold increase in *Noggin* gene expression after 4 and 18 hours, respectively. **Figure *21***

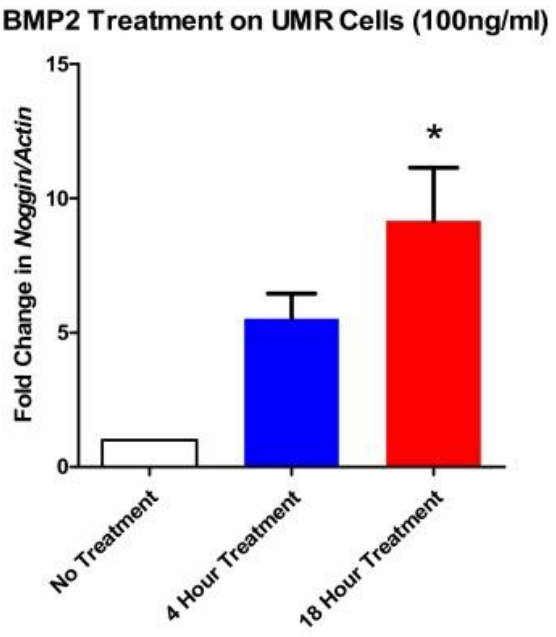


Figure 21. *rhBMP2 treatment on UMR cells induces the* Noggin *response - Treatment with hBMP2 triggered the negative feedback response and resulted in a 5-fold and 9-fold increase in the expression of Noggin after 4 and 18 h, respectively. (n = 3) (Mean ± S.D. * p < 0.05).*

3.4. siRNA treatment and cell toxicity

Live-dead assay with cell treated with Lipofectamine- siRNA showed, on average, 20 dead cells out of 700 cells after 24-hour treatment. The cell viability percentage was not statistically significantly different between three treatment groups of Lipofectamine alone, siRNA alone or the combination of Lipofectamine-siRNA. **Figure *22***

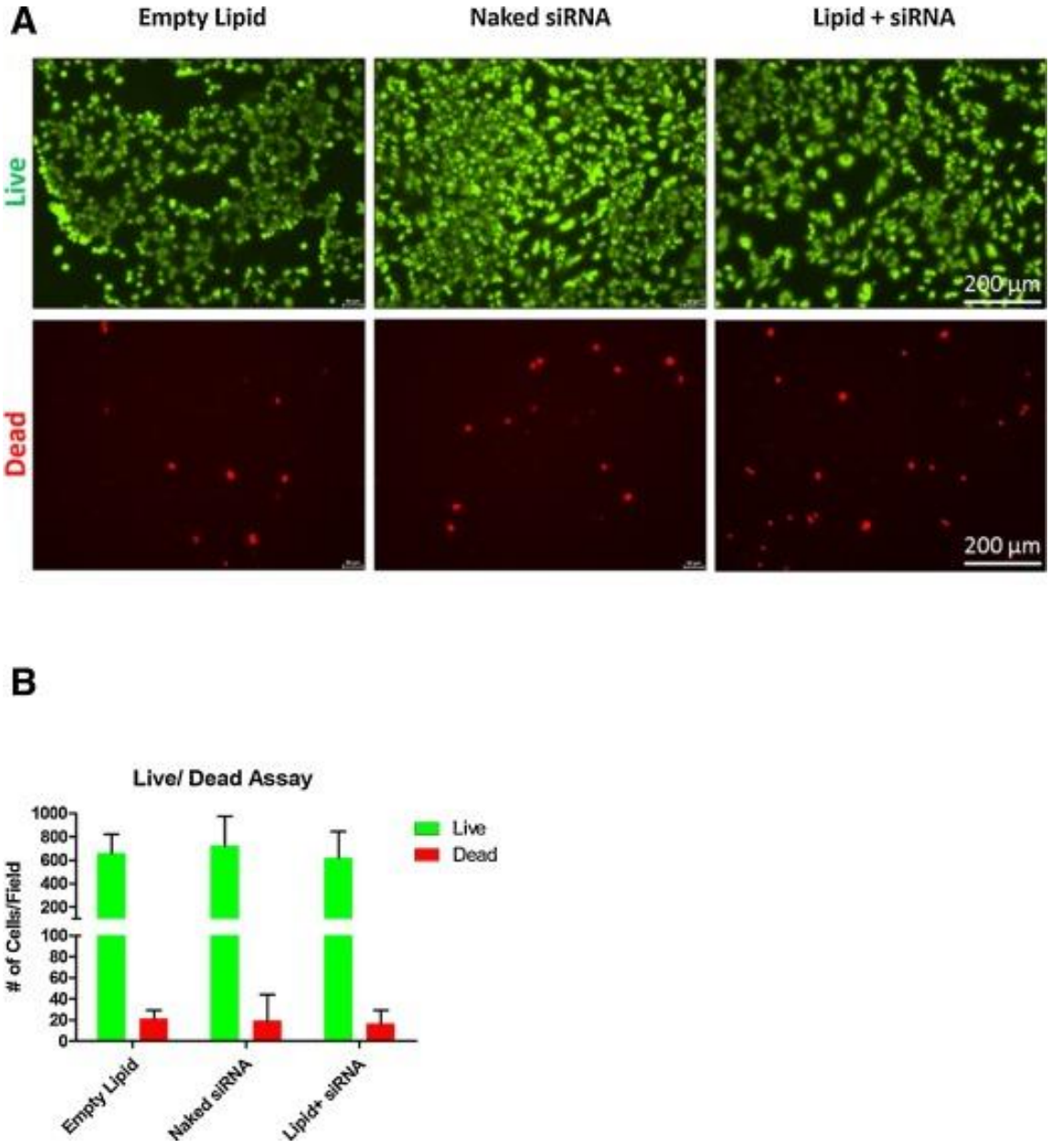


Figure 22. *Lipid nano-particle delivery of siRNA and cell toxicity* **-** *UMR cells were treated with only lipid nano particle, only siRNA or the combination of Lipid-nanoparticle and siRNA.* **A.** *LIVE/DEAD® Viability/Cytotoxicity assay revealed similar results in all three groups with minimal cell death (bottom panel, red)* **B.** *The majority of the cell population survived 24 h after the treatment in all three treatment groups. (n = 3, cells in six microscope fields were counted and averaged in each sample) (Mean ± S.D.).*

3.5. siRNA treatment and transfection efficiency

The cell transfection efficiency of LNP-siRNA complex in osteoblast cells was tested by treating cultured UMR-106 cells with a Lipofectamine- fluorescently labeled negative siRNA and confocal microscopy was performed **Figure *23***A. Cell soring was performed on three groups of treatment with naked fluorescently labelled siRNA, Lipofectamine alone or the combination of Lipofectamine- fluorescently labeled siRNA. Treatment with 50nM fluorescently labeled negative control siRNA resulted in over 85% transfection efficiency in the combination treatment group **Figure *23***B.

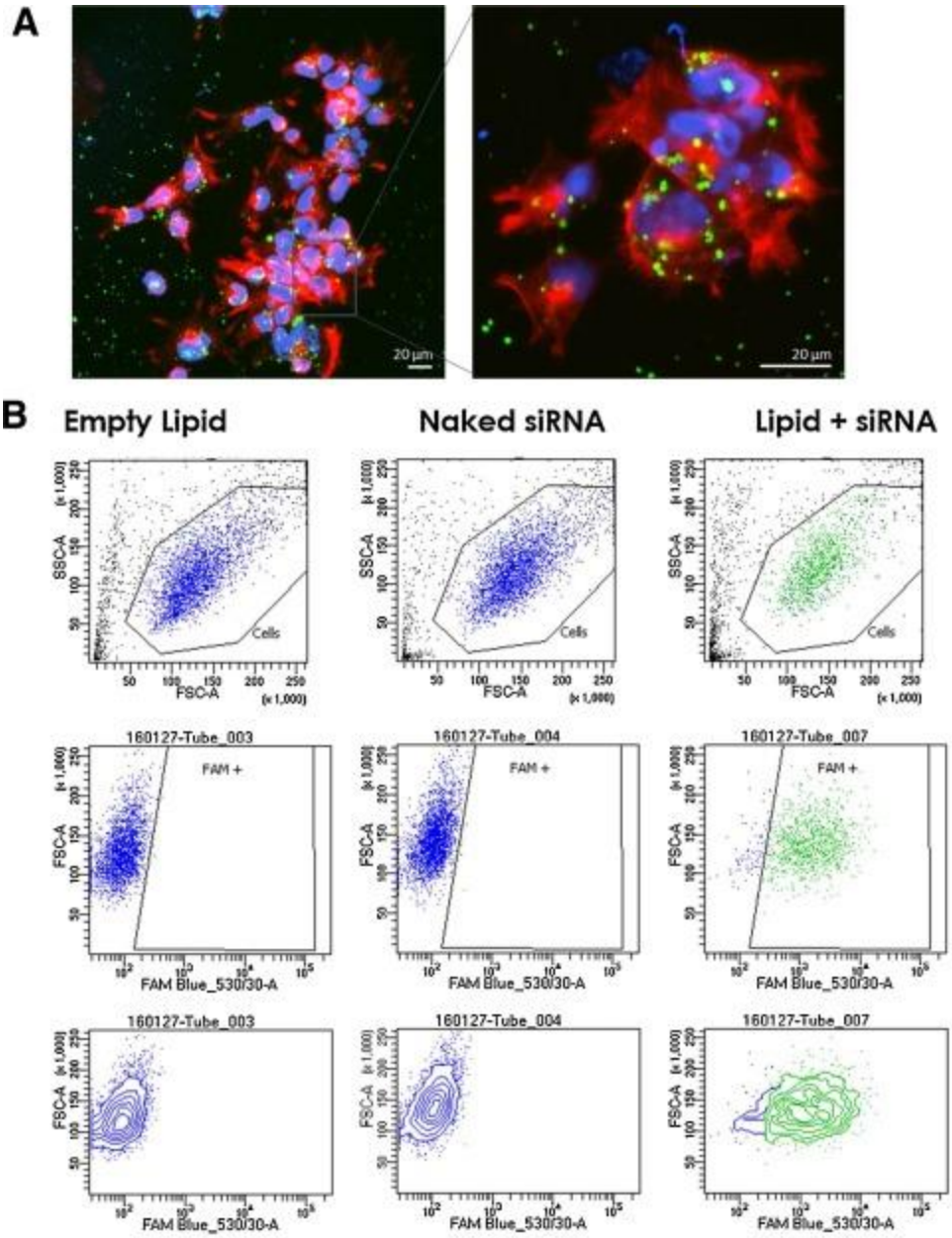

A
20 µm
20 µm
B
Empty Lipid
Naked siRNA
Lipid + siRNA
SSC-A
FSC-A
Cells
160127-Tube_003
160127-Tube_004
160127-Tube_007
FAM +
FAM Blue_530/30-A
C
% FAM+/Total
100
80
60
40
20
0
*
Empty Lipid
Naked siRNA
Lipid+ siRNA

Figure 23. *Cell transfection efficiency -* **A.** *cultured UMR-106 cells were treated with a Lipofectamine- fluorescently labeled negative siRNA, confocal microscopy: Red: actin, Blue: nucleus, Green: siRNA.* **B.** *Cell sorting was performed based on the siRNA florescent tag: FAM.* **C.** *The graph shows the percentage of FAM^{+} cells over total number of cells counted.*

3.6. Noggin siRNA screening

We then screened a library of three different Noggin siRNAs -referred to as Noggin siRNA 1, 2 and 3- to find the most effective Noggin siRNA capable of down-regulating *Noggin* gene expression. UMR-106 cells initially treated with rBMP-2 were transfected with different Noggin siRNAs, then we checked the *Noggin* gene expression after 24 hours. All Noggin siRNAs (1, 2 and 3) reduced the *Noggin* gene expression significantly compared to the negative control siRNA, and Noggin siRNA 1 was identified to be effective in over 60% *Noggin* gene knock-down after 24 hours treatment, confirmed by RT-qPCR. This Noggin siRNA (Nog 1) was used for the rest of our experiments. **Figure *24***

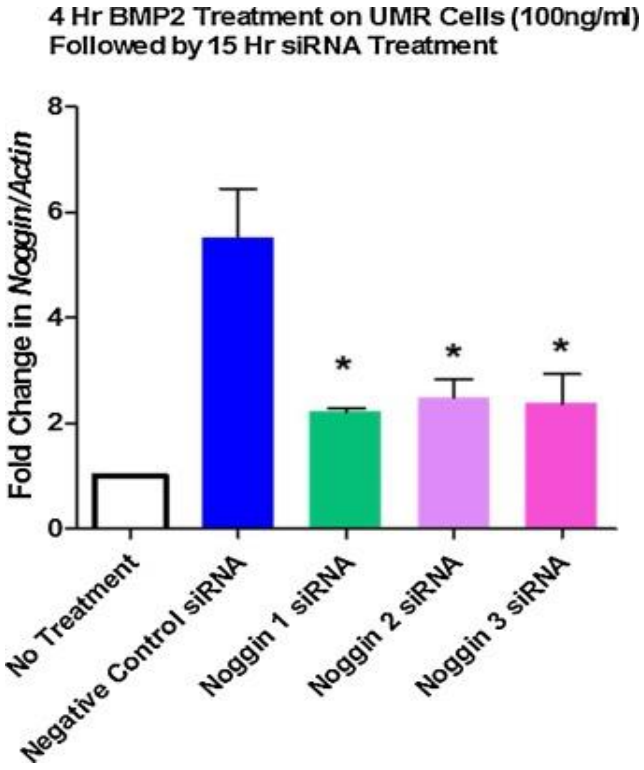


Figure 24. *Noggin siRNA screening. A library of three different Noggin siRNAs was screened to find the most effective one in down-regulating the* Noggin *expression. The graph shows the results of RT-qPCR assay for* Noggin*expression. UMR-106 cells initially treated with hBMP-2 were transfected with different Noggin siRNAs,* Noggin *gene expression was quantified after 24 h and compared to the negative control siRNA. Noggin siRNA 1 was identified to be effective in over 60%* Noggin *gene knock-down after 24 h treatment. (n = 3) (Mean ± S.D.* p < 0.05).

3.7. Nog1 siRNA: Noggin protein knock down

Functionality of Nog 1 siRNA was tested *in-vitro* by measuring the Noggin protein levels 48 hours after treatment with this siRNA in culture. Protein expression analysis by

sandwich enzyme immunoassay performed using a rat Noggin-specific ELISA assay (Cloud-Clone Corp.®) confirmed a 70% decline in Noggin protein expression in response to Nog 1 siRNA treatment after 48 hours. **Figure *25***

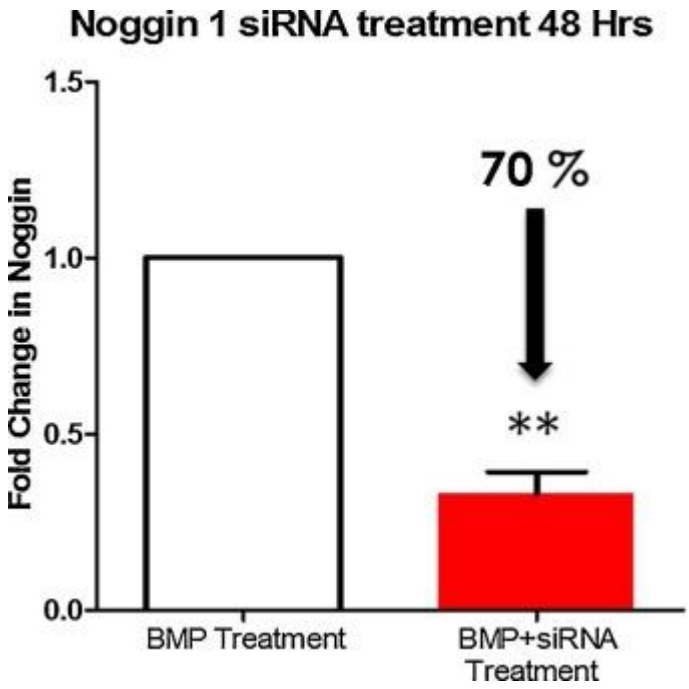


Figure 25. *Noggin protein knock down. Noggin protein levels were measured 48 h after treatment with Noggin 1 siRNA in culture. Sandwich enzyme immunoassay performed using a rat Noggin-specific ELISA assay (Cloud-Clone Corp.®) confirmed a 70% decline in Noggin protein expression in response to Nog 1 siRNA treatment after 48 h. (n = 3) (Mean ± S.D. * p < 0.01).*

3.8. Other BMP signaling inhibitors

To determine whether the inhibition of Noggin would trigger a change in the activity of other BMP signaling inhibitors i.e. Gremlin and Chordin, the expression of

Gremlin and *Chordin* in response to the treatment with Noggin siRNA (Nog 1) was investigated to explore any down-regulation or compensatory up-regulation response from these inhibitors. Pre-treatment with rhBMP-2 was able to up-regulate the expression of these negative regulators of BMP signalling, however, interestingly, the same treatment with Nog-1 which led to a significant reduction in Noggin expression, did not cause any statistically significant change in the expression of either *Gremlin* or *Chordin* after 24 hours of siRNA treatment. **Figure *26***

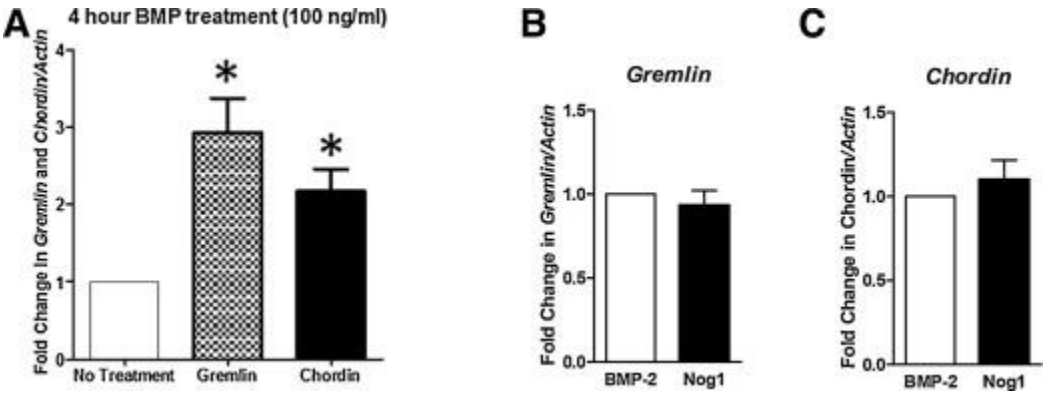


Figure 26. *Other BMP signaling inhibitors.* **A.** *Pre-treatment with 100 ng/ml hBMP-2 was performed.* **B & C.** *The expression of* Gremlin *and* Chordin *in response to the treatment with Noggin siRNA (Nog 1) was investigated after 24 h to explore any down-regulation or compensatory up-regulation response from these inhibitors. (n = 3) (Mean ± S.D. * p < 0.05).*

3.9. Upregulation of downstream targets of BMP signal

The expression of direct BMP-2 downstream genes (*Smad 1&5*) and also osteoblast-specific genes after siRNA treatment were quantified to study the effects of

Nog 1 siRNA on BMP downstream genes and osteoblastogenesis. Treatment with Nog 1 siRNA induced a significant increase in BMP-2 downstream targets *Smad 1* and *Smad 5* and resulted in an upward trend in the expression of *Bsp* and *Runx2*, only 24 hours after treatment. **Figure *27***

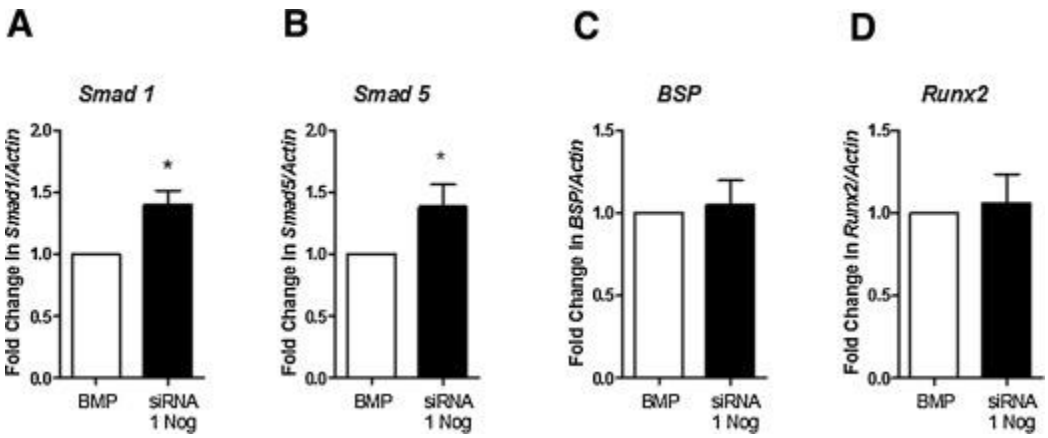


Figure 27. *Upregulation of downstream targets of BMP signal.* ***A&B.*** *The expression of direct hBMP-2 downstream genes (Smad 1&5) and* ***C&D.*** *osteoblast-specific genes after siRNA treatment were quantified using RT-qPCR assay to study the effects of Nog 1 siRNA on BMP downstream genes and osteoblastogenesis. (n = 3) (Mean ± S.D. * p < 0.05).*

3.10. Osteogenesis and matrix mineralization

To investigate the effectiveness of Nog1 siRNA in inducing the osteogenesis in culture, we designed another experiment to investigate osteoblast differentiation by measuring the activity of the specific early osteoblast differentiation marker, alkaline phosphatase (ALP) (Marom, Shur et al. 2005), and also the amount of mineralized matrix formation by the osteoblasts in culture. Cultured UMR-106 cells, treated with osteoblastic medium and Nog 1 siRNA, received low doses of rBMP-2 and the

mineralized matrix formation was quantified after 10 days of culture by Alizarin Red staining assay. Treatment with Nog 1 siRNA resulted in a 2.5 fold increase in Alp activity **Figure *28***A and over 3 fold increase in mineralized matrix formation, compared to the BMP-2 treatment group, confirmed by Alizarin Red assay. **Figure *28*** B&C

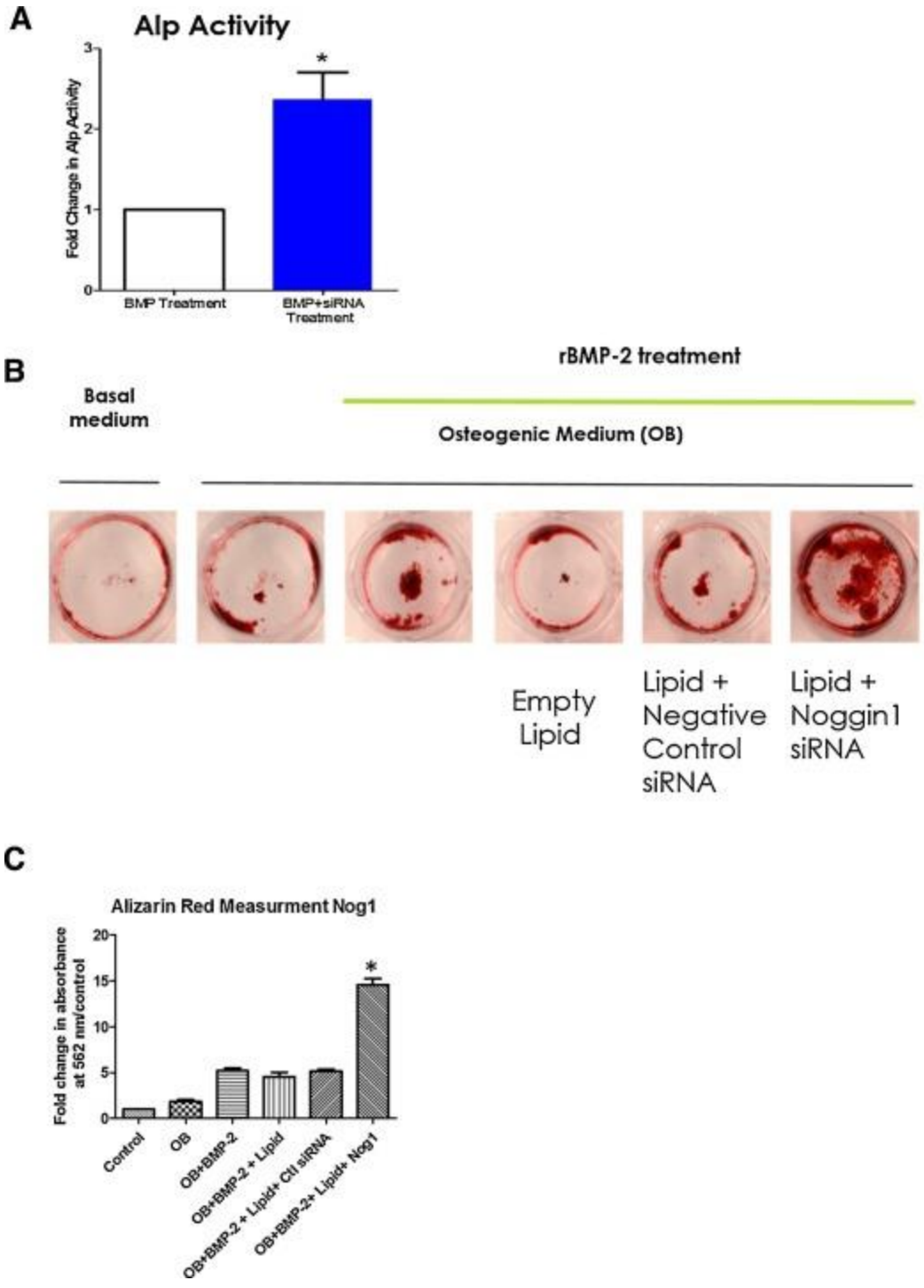


Figure 28. *Osteogenesis and matrix mineralization.* **A.** *ALP activity measured by an ALP-specific enzymatic test on UMR cells treated with hBMP-2 and Noggin siRNA 1 for 7 days (n = 3) (mean ± S.D.** p *< 0.05)* **B.** *Alizarin Red staining of cultured UMR cells*

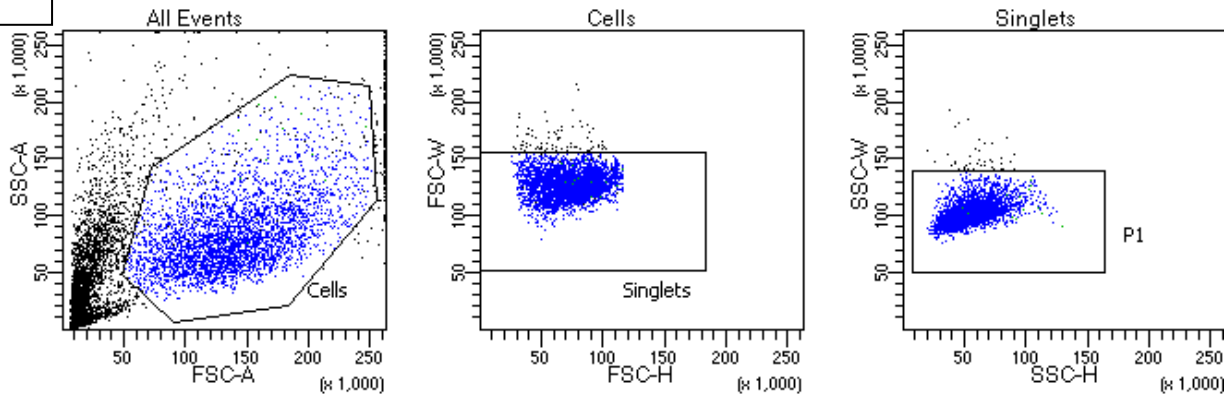
with different treatments. **C.** *Quantification of Alizarin Red stain. Treatment with Noggin siRNA, significantly enhances hBMP-2 efficiency and increases osteoblast mineral formation.*

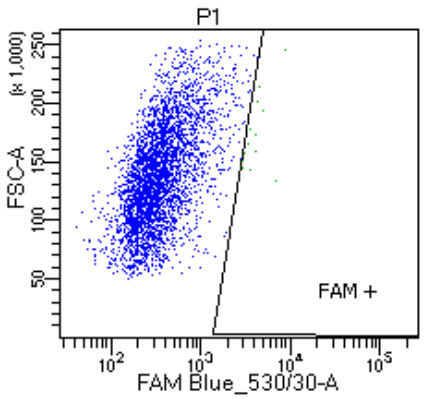
3.11. Trial on human bone cells

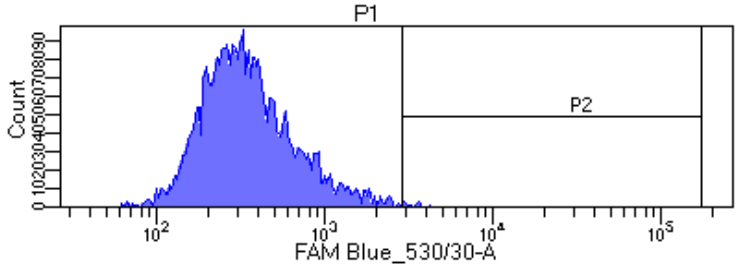
The same siRNA transfection used on UMR cells was employed to test on primary bone cells derived from healthy human samples. This experiment was carried out as a trial and proof of concept to test if our successful methodology had the potential to yield comparable results on human bone cells from a translational research perspective.

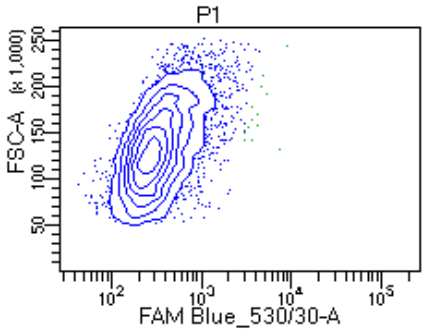
The followings are representative FACS results from three treatment groups: A: Cells treated with Lipofectamine only, B: Cells treated with siRNA only and C: Cells treated with LNP-siRNA. The preliminary results demonstrate a transfection efficiency of over 85% using the control siRNA-FAM and Lipofectamine 2000.

A









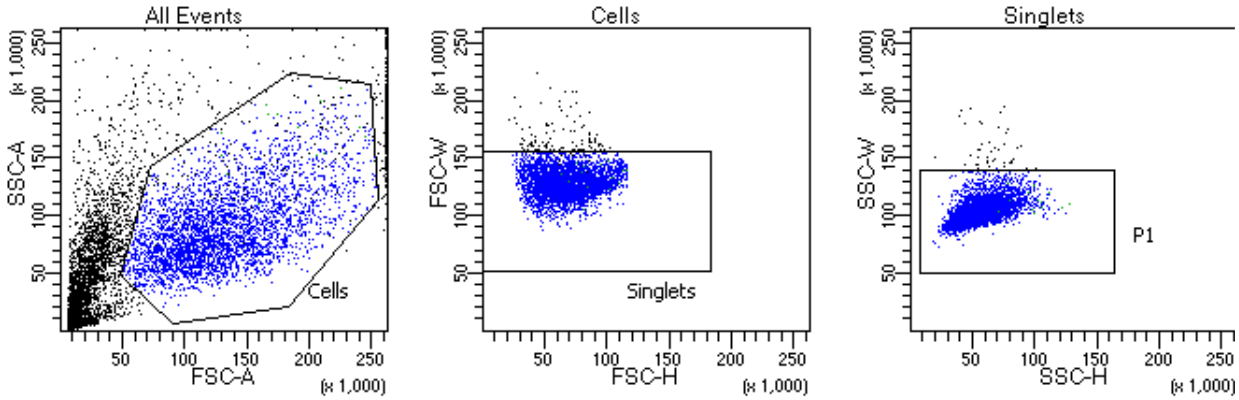
Tube: Tube_001

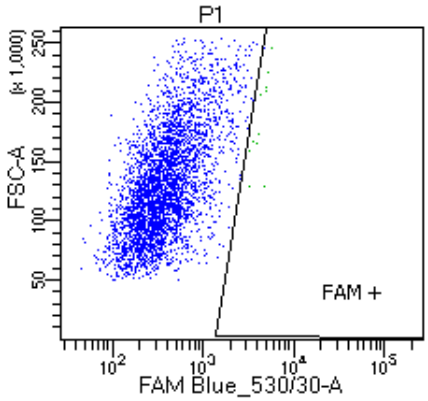
Population	#Events	%Parent	%Total
All Events	7,997	####	100.0
Cells	3,631	45.4	45.4
Singlets	3,555	97.9	44.5
P1	3,514	98.8	43.9
FAM +	13	0.4	0.2
P2	27	0.8	0.3

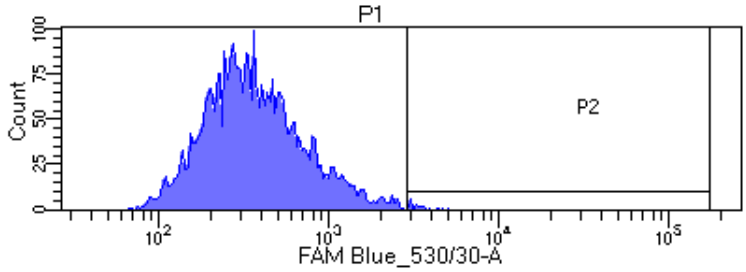
Experiment Name:	Saber_FAM_FACS_160120
Specimen Name:	160527
Tube Name:	Tube_001
Record Date:	May 27, 2016 4:45:41 PM

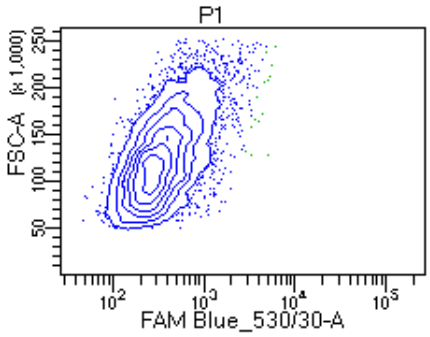
Population	#Events	%Parent	FSC-A Mean	FAM Blue_... Mean
P1	3,514	98.8	138,136	448
FAM +	13	0.4	174,006	4,331
P2	27	0.8	194,215	3,784

B









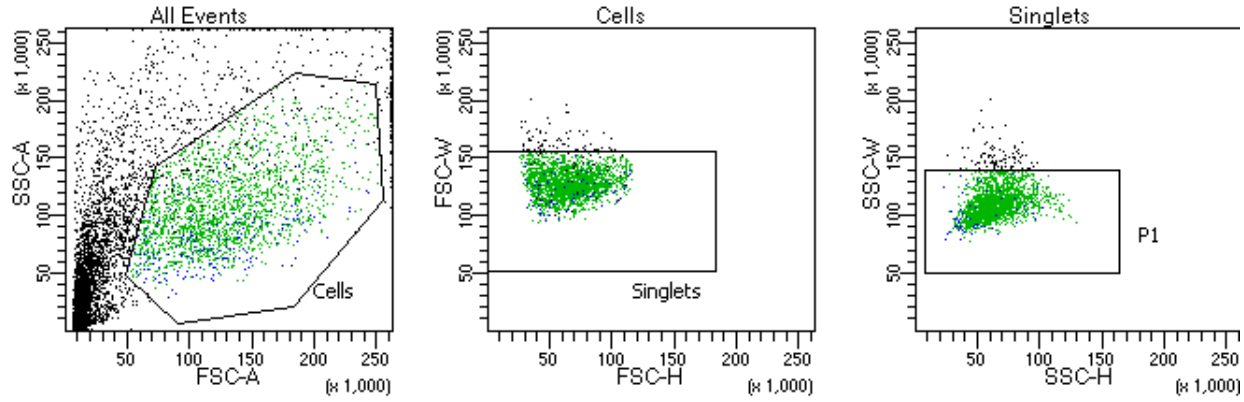
Tube: Tube_009

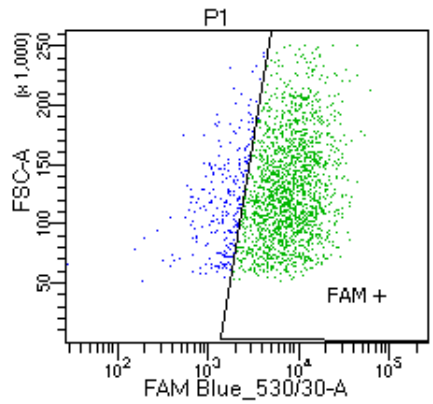
Population	#Events	%Parent	%Total
All Events	8,016	####	100.0
Cells	3,771	47.0	47.0
Singlets	3,667	97.2	45.7
P1	3,624	98.8	45.2
FAM +	14	0.4	0.2
P2	37	1.0	0.5

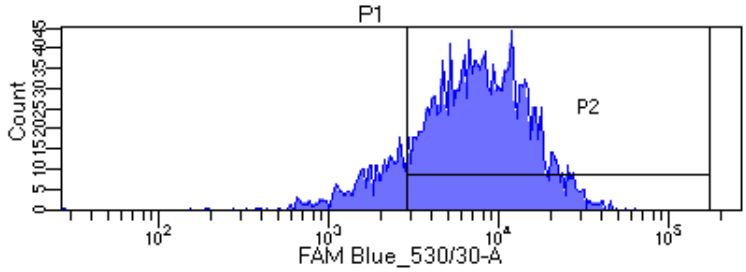
Experiment Name:	Saber_FAM_FACS_160120
Specimen Name:	160527
Tube Name:	Tube_009
Record Date:	May 27, 2016 5:31:03 PM

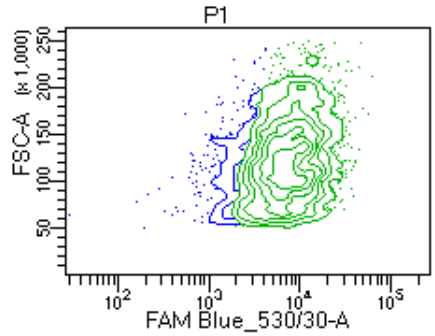
Population	#Events	%Parent	FSC-A Mean	FAM Blue_... Mean
P1	3,624	98.8	130,530	484
FAM +	14	0.4	184,905	4,142
P2	37	1.0	207,919	3,626

C









Tube: Tube_012

Population	#Events	%Parent	%Total
All Events	6,553	####	100.0
Cells	2,127	32.5	32.5
Singlets	2,057	96.7	31.4
P1	1,965	95.5	30.0
FAM +	1,709	87.0	26.1
P2	1,655	84.2	25.3

Experiment Name:	Saber_FAM_FACS_160120
Specimen Name:	160527
Tube Name:	Tube_012
Record Date:	May 27, 2016 5:09:38 PM

Population	#Events	%Parent	FSC-A Mean	FAM Blue_... Mean
P1	1,965	95.5	128,143	8,544
FAM +	1,709	87.0	129,128	9,576
P2	1,655	84.2	131,767	9,820

Figure 29. *FACS results demonstrating a high transfection efficiency of Lipofectamine 2000 utilizing siRNA-FAM on cultured human osteoblasts.* ***A.*** *Control 1: siRNA-FAM without the LNP carrier* ***B.*** *Control 2: Lipofectamine alone, without siRNA-FAM and* ***C.*** *LNP-siRNA-FAM*

3.12. Trial of a collagen scaffold for LNP-siRNA delivery

To study the properties of a collagen hydrogel/scaffold for noggin-siRNA delivery to bone defects, we prepared Collagen Type 1 scaffolds from rat tail Col-1.

For the purpose of this experiment, the mixture of Lipofectamine-siRNA was prepared according to the protocols previously used on cultured cells. Instead of adding this cocktail to the cultured cells, we first detached the cells and rinsed them with PBS. The cells were resuspended in normal growing medium (DMEM) with 10% FBS and the volume of this mixture was topped with extra medium. The collagen gel was diluted with varying volumes of the final solution containing all the transfection reagents to optimize the methodology.

Below are different experimental groups:

1: 30% Gel, 70% Cells+ siRNA+ Lipofectamine in Medium

2: 50% Gel, 50% Cells+ siRNA+ Lipofectamine in Medium

3: 75% Gel, 25% Cells+ siRNA+ Lipofectamine in Medium

4: 100% Gel

5: 50% Gel, 50% Lipofectamine in Medium

The gels were kept in sterile tissue culture hood 37 C and 5% CO2 for either 24 hours or 10 days of culture and the events were monitored during the culture time (to check for contamination, shrinkage, etc.).

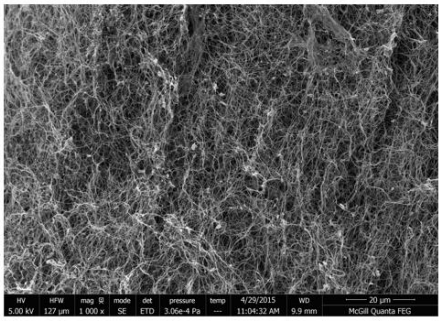
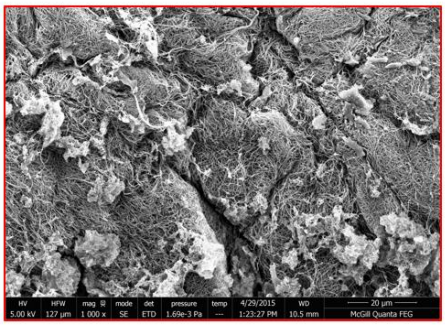
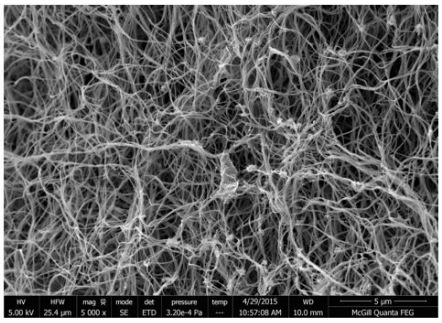
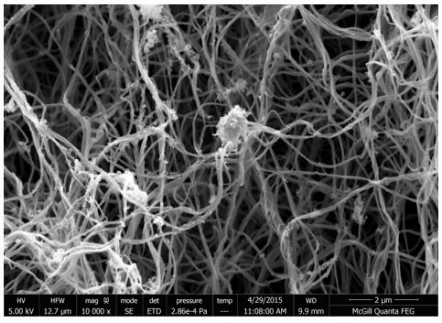
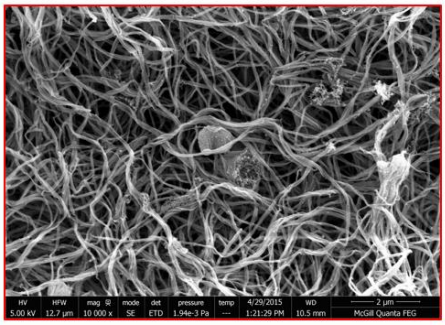
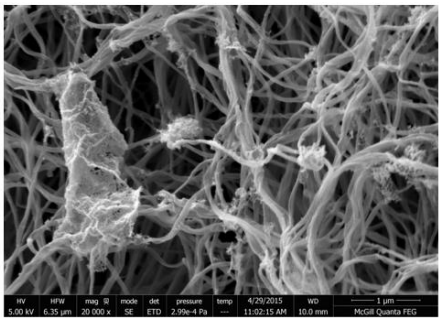
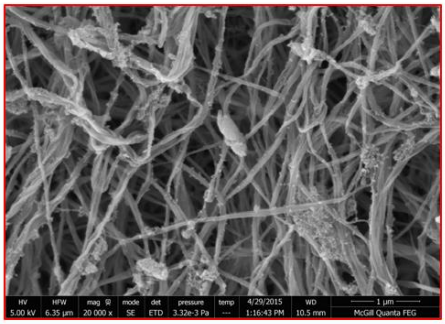
Following each timepoint, the samples were fixed using 10 % formaldehyde and sent to a collaborating lab for electron microscopy analysis. Unfortunately, some samples were misplaced during the process in the other lab.

Initially we planned to use a new microscope to get electron microscopy and confocal imaging simultaneously, however, the new technique did not become available. This would have enabled us to study the structure of our scaffolds and at the same time, localize the siRNA transfected cells, utilizing the fluorescent label of our siRNA.

We performed Scanning Electron Microscopy (SEM) only on samples from groups 1, 2 and 4; hereafter called Groups A (30% Gel), B (50% Gel) and C (100% Gel) respectively.

Below are the SEM images from the above groups (**Figure 33**):

- 70% Cells+ siRNA+ Lipofectamine+ Medium
- 30% Collagen Gel

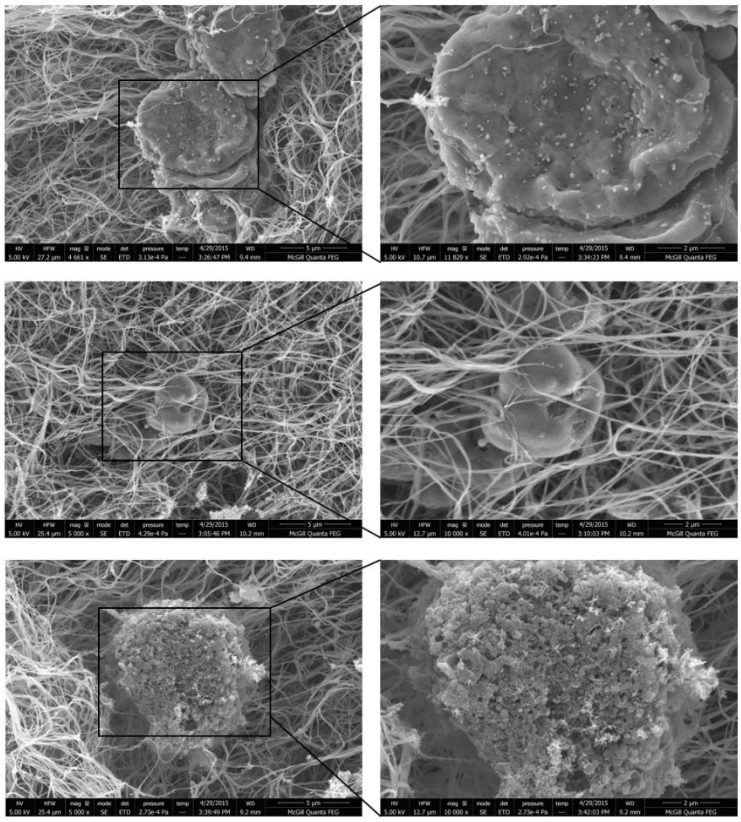
A 1 (After 24 Hrs)	A 2 (After 10 days)
	
	
	
	

- 50% Cells+ siRNA+ Lipofectamine+ Medium

- 50% Collagen Gel

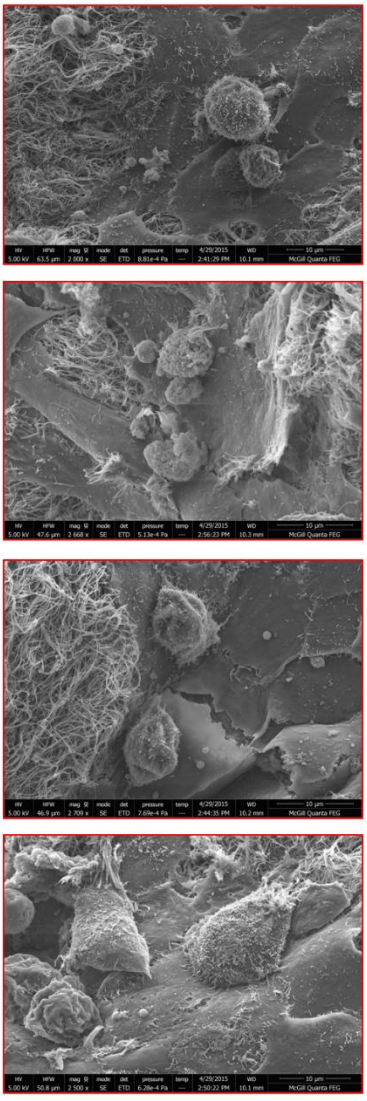
B 1

(After 24 Hrs)



B 2

(After 10 days)



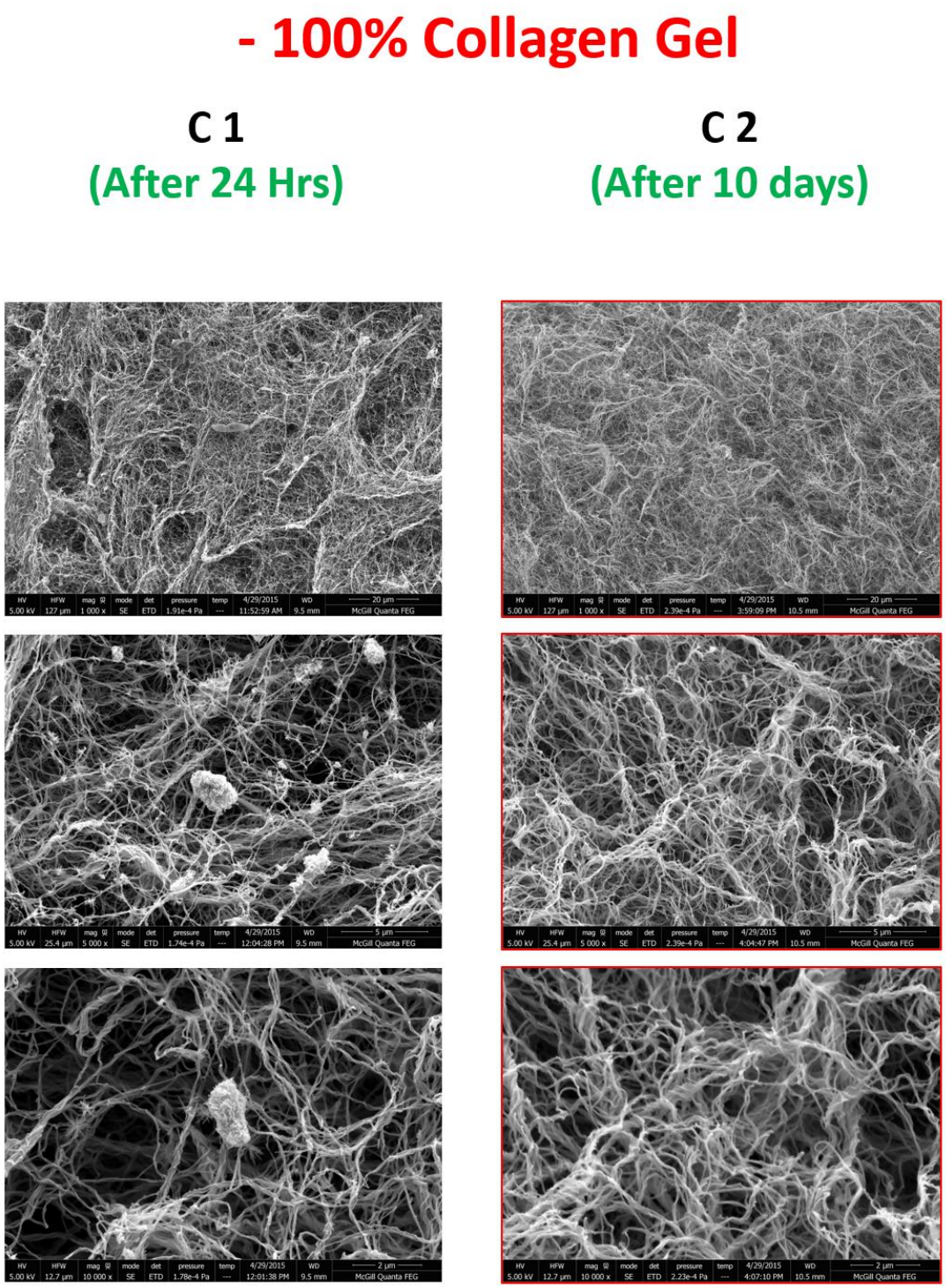


Figure 30. *Electron Microscopy images of collagen scaffolds with different formulations*

and in two different timepoints at 24 hours and 10 days after culture.

In each of the above SEM images, the scale bar (calibrated in μm) is shown on the lower right of the digital image for reference.

In all images, the expected ridged structure of collagen fibers is visible. Without proper and accurate quantification and analysis, we cannot comment whether the gel has degraded after 10 days or not, however, this phenomenon is not obvious in the images.

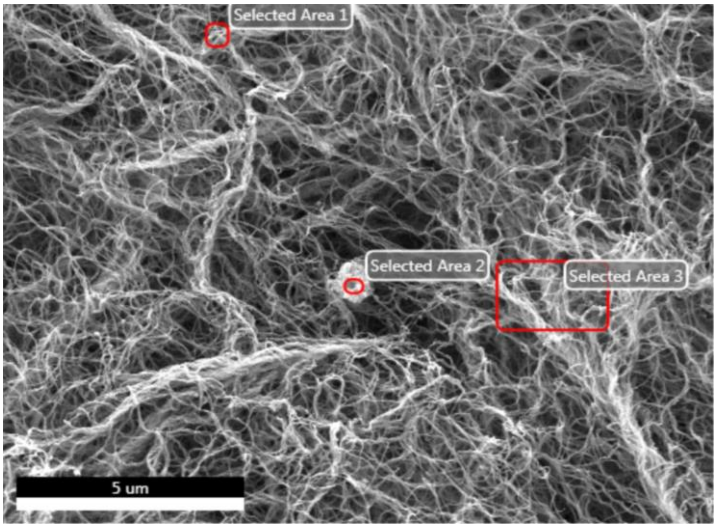
Additionally, in groups A and B, the cells are visible, incorporating into the collagen gel; however, solitary cells in the left column, which are representative images of the gels 24 hours after the start of the experiment, are smaller (Note: different scale bars), without obvious cell adhesion and penetration inside the gel. Conversely, 10 days after, the cells seem to be larger and penetrating much more into the gel pores with more matrix formation. Interestingly, a larger number of cells with significantly more cell processes are seen in group B with 50% collagen gel. Perhaps this 50% concentration can provide an environment which is structurally more convenient for the cells to grow in. This needs to be further confirmed through future experiments.

On the surface of the cells, as well as within the gel, there are clumps of white material, dissimilar to the rest of the collagen fiber structure. These could represent the Lipofectamine liposomes, however, this needs to be further assayed with better control groups.

In some images, star-shaped crystalline structures were found (**Figure 31**) and we further analyzed some 10 day images using Energy Dispersive X-Ray (EDX)

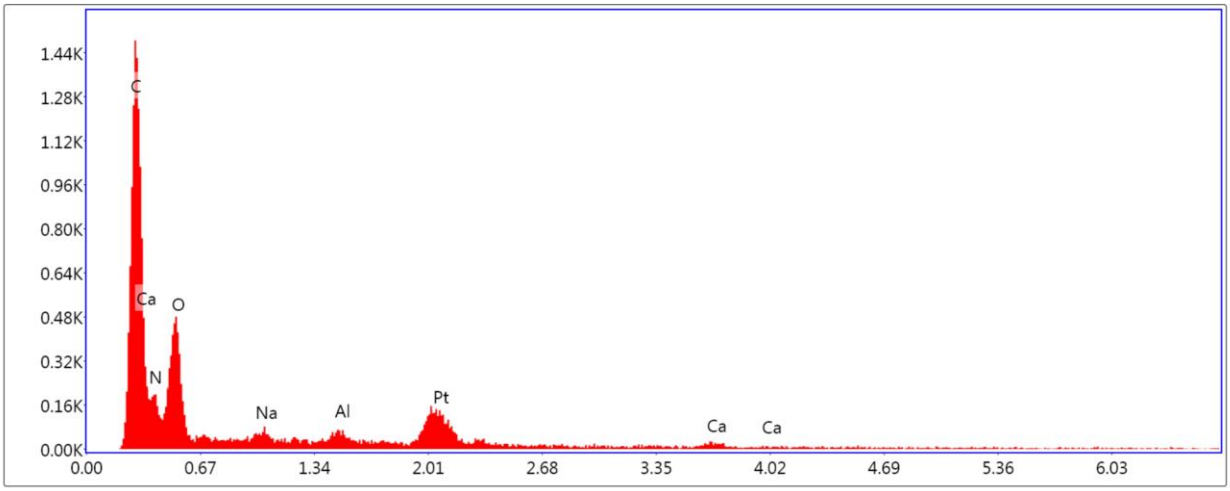
spectroscopy to check the comprising elements in those areas and compare them with neighboring regions for potential differences. The results are shown below, however, no significant difference in the estimated comprising elements was found in these areas compared to the cell surface of collagen fibers.

Group C, 100% Gel only:



Selected Area 1

Selected Area 2



Selected Area 3

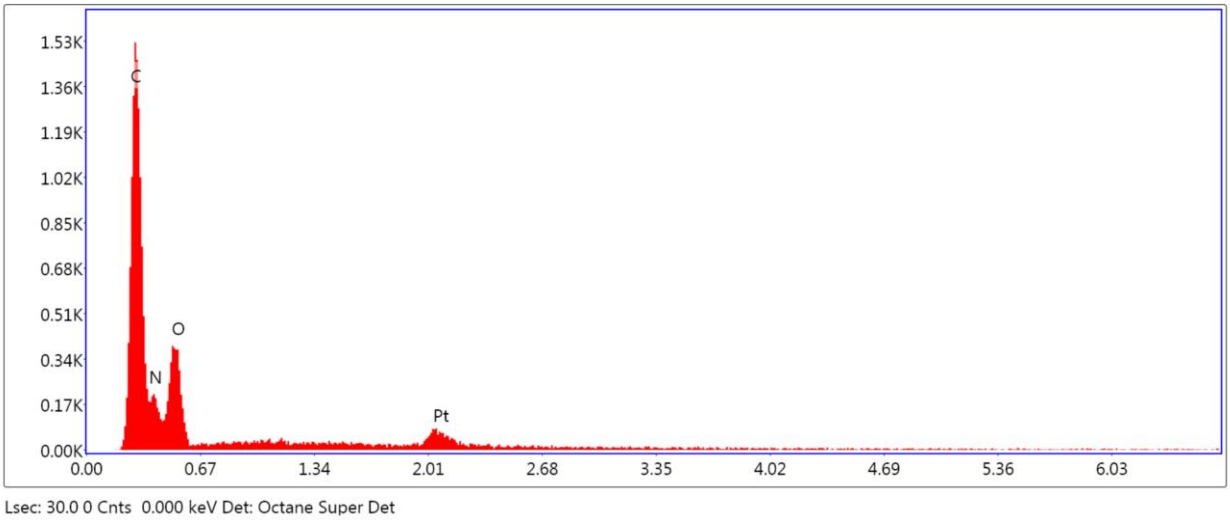


***Figure 31**. Representative image shows the star-shaped crystalline structure that was found in some SEM images. Analysis using Energy Dispersive X-Ray (EDX) spectroscopy showed no significant differences in composition with two neighboring regions.*

3.13. *In vivo* trial- proof of concept

Our *in vitro* results demonstrated that LNP- Noggin siRNA treatment has a stimulatory effect on the osteogenic differentiation and mineralized matrix formation of UMR bone cells. Following these *in vitro* experiments with satisfactory results, we next aimed to examine the feasibility of utilizing the LNP-siRNA system incorporated in a collagen scaffold in an animal model of fracture healing. Bone grafts are used for several clinical applications in orthopedic surgery. 3D porous scaffolds are effective in both cell targeting and cell movement strategies. These 3D scaffolds offer surfaces that enable cell attachment, survival, migration, proliferation, and differentiation, as well as an appropriate 3D area in which neovascularization, tissue growth, and remodeling is possible. The main concern with such an approach using our system was the degradation of oligonucleotides *in vivo* in contact with the enzymes released from animal tissue. Another biological and clinical risk associated with such treatments would stem from the diffusion of active agents within the 3D scaffold to neighboring areas of the bone defect, resulting in off target effects such as ectopic ossification and interference with the normal growth of unaffected tissue. As such, an ideal scaffold delivering therapeutic agents to the area of fracture or non-union, would remain locally and last long and non-degraded enough to actively contribute to the slow tissue regeneration process.

To assess the properties of the current system incorporated in a collagen scaffold, we used fluorescently labelled control oligonucleotides to monitor their

diffusion from the initial application location.

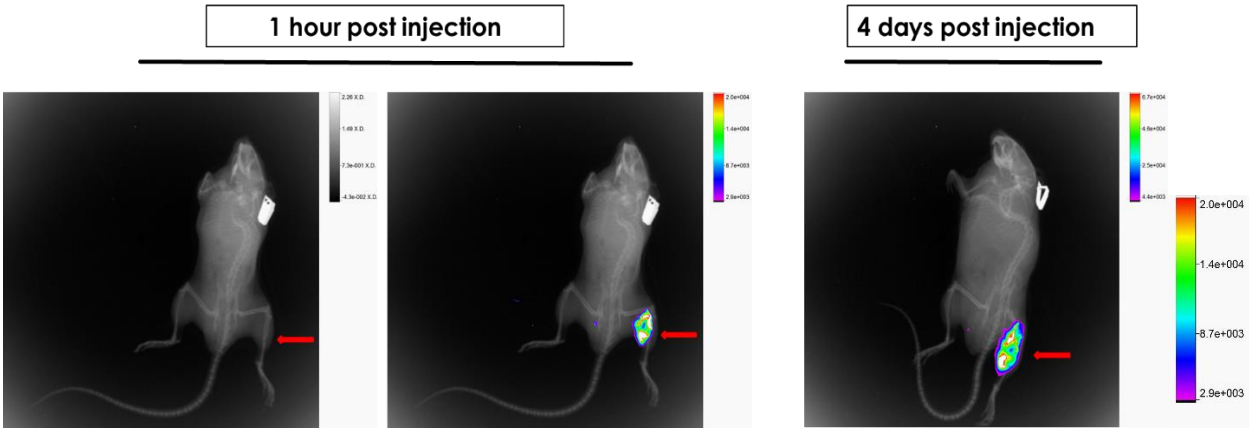


Figure 32. *Fluorescent imaging of treated fractured tibia with fluorescently labelled oligonucleotide seeded onto collagen scaffolds and subcutaneously implanted at the site of tibial fracture mice captured using an In-Vivo Xtreme system imaging machine in two different time points, 1 hour and 4 days post injection.*

As shown in the above picture (**Figure 32**), one hour after the initial injection, the injected scaffold containing florescent oligonucleotide is detected (Left) locally at the site of fracture (Red arrow). The same animal 4 days later is tested and again, the treatment appears to be located in the same area, without noticeable diffusion and signals similar intensity of florescence.

Nevertheless, as the fluorescent moiety attached to the oligonucleotide would still be detected even after it is detached from the functional sequence, detection of florescent signal cannot prove the wholeness of the moiety and the oligonucleotide system and as such, it is difficult to comment on the degree of degradation.

4. Discussion

“Despite the critical role of BMPs in accelerating bone formation in several orthopedic procedures, there has been growing evidence attributing various adverse events to the use of supra-physiologic doses of exogenous BMP in clinic (Haidar, Hamdy et al. 2009, Haidar, Hamdy et al. 2009, Evans 2010, Epstein 2013).” As presented in the introduction of current thesis, recent systematic reviews on this topic have emphasised and highlighted the unwanted effects of exogenous BMP, particularly BMP-2 and BMP-7, among patients. The spectrum of negative side effects expands from local irritation and medication allergy to serious life-threatening adverse events such as infection exacerbation and ectopic/heterotopic bone formation which could cause blocking the blood circulation and/or nerve impingement with excruciating pain. Despite some promising results from clinical application of such auxiliary treatments in the management of bone fractures, exogenous BMP treatment cannot be considered a safe and effective treatment for these orthopedic conditions.

“This highlights the need for an alternative approach towards promoting physiologic bone repair. Noggin is a known target to achieve increased BMP efficacy as it is a key extracellular BMP antagonist (Canalis, Economides et al. 2003, Chen, Zhao et al. 2004) and its inhibitory role in BMP-mediated osteogenesis has been extensively investigated both in animal models and rodent cells(Gazzerro, Gangji et al. 1998, Abe, Yamamoto et al. 2000). Several studies on animal models have exhibited the effectiveness of blockage of Noggin and consequent increase in BMP levels (Abe, Yamamoto et al. 2000, Wan, Pomerantz et al. 2007, Takayama, Suzuki et al. 2009, Klineberg, Haudenschild et al. 2014). Nevertheless, previous studies utilized viral vector-mediated gene silencing methods which lack realistic clinical translatability and

raise serious safety concerns for clinical applications. In the current study, we aimed to test a natural and safe lipid-based delivery carrier for siRNA which bears the potential for actual clinical application and utility and as far as we are aware, this strategy has not been investigated previously on rat or human osteoblastic cells.

Treatment with recombinant BMP-2 resulted in a significant up-regulation of Noggin very quickly. This is perhaps what contributes the most to low efficiency of exogenous BMP-2 treatment in clinic, a huge negative feedback signal competing with BMP-2 and necessitating higher doses. All of Noggin siRNAs that we tested were capable of efficient Noggin inhibition, however, we chose the most effective one and continued the experiments using that. Down-regulation of Noggin resulted in a significant increase in the expression of key downstream genes of BMP signalling and in the osteoblastic differentiation markers such as Alp. Furthermore, mineralized matrix formation by UMR cells was significantly enhanced in the Noggin siRNA treated groups. Extracellular matrix mineralization was detected by Alizarin Red staining as early as 10 days of culture in BMP-2 and Noggin siRNA treated group. This confirms the synergic effect of minimal doses of rBMP-2 and siRNA-mediated Noggin inhibition on bone regeneration and mineral formation.

The levels of Gremlin and Chordin were similarly unchanged in response to Noggin siRNA treatment and this suggests the specific targeting of the siRNA and no compensatory response from the other BMP signaling inhibitors. To the best of our knowledge, this aspect has never been investigated previously and the effects of Noggin inhibition on other BMP signal inhibitors was not clear. Our results revealed that not only is the Noggin inhibition via siRNA very specific, this inhibition does not trigger

any compensatory inhibition by other BMP inhibitors. Nonetheless, these results provide basic evidence of the interaction between Noggin, Chordin and Gremlin pathways and further research is needed to elucidate these interactions in animal models of bone regeneration.

Cell toxicity assays showed very limited harmful effects of LNPs on the cultured cells. The development of new lipid delivery vehicles is an ever-changing area of research that constantly introduces novel carriers for drug delivery.

Recent advancements in the area of lipid based drug delivery methods have led to the development of interesting LNPs which enhance the drug stability, improve bioavailability and minimize drug degradation (Li, Dai et al. 2008).One type of novel LNPs that has gained substantial attention by clinicians and proves to be suitable for orthopaedic applications is the layer-by-layer LNP consisting of natural biodegradable polymers. Our group and others have developed and characterized various LNPs with a lipid core and alginate-chitosan layers (Douglas and Tabrizian 2005, Choi, Kim et al. 2013, Ghadakzadeh, Mekhail et al. 2016, Nayef, Castiello et al. 2017).

L-B-L formulations possess interesting features which can encapsulate multiple therapeutic agents within different layers. Utilizing these nanoparticles would allow the slow release of small nontoxic amounts of different types of BMPs and other growth factors simultaneously or chronologically over the course of bone repair. However, our experiments revealed that this type of LNPs are not favorable for siRNA delivery. The results from encapsulation and cell transfection efficiency experiments showed that Chitosan- Alginate L-B-L LNPs were not successfully delivering the siRNA to the region of action, which for siRNA is the cytoplasm. Successful delivery of BMPs is not

dependent on delivery of the cargo all the way to the intracellular space; the mechanism of action of such growth factors, activating the BMP signaling pathway as a result of extracellular attachment of the ligand (BMP molecules) to their specific cell surface receptors, could be perfectly achieved by means of L-B-L LNPs. Furthermore, the fact that cell internalization would not occur efficiently with the Chitosan- Alginate L-B-L LNPs (due to low cationic charge or relatively large size of the particles), could act in favor of efficient BMP delivery; slow release of tiny amounts of BMP from the particles stuck in the extracellular space would generate a consistent positive trigger to up-regulate the BMP signaling over the course of bone repair."

"siRNA-based therapies have been gaining much attention and their use in a wide range of applications, including bone regeneration, has been promising. However, there are some universal challenges facing the use of siRNA therapeutics. Almost a decade ago, three main off-target effects of siRNA were discovered, with potential unwanted side effects (Jackson and Linsley 2010). The first is silencing a number of unintended genes through partial sequence complementarity; second is inflammatory response caused by either the siRNA or DDS used; and third is saturating the endogenous RNAi machinery, which could affect normal miRNA functionality (Jackson and Linsley 2010). In order to avoid silencing unintended genes, there has been evidence that lowering the concentration of siRNA used can significantly reduce these unwanted effects (Caffrey, Zhao et al. 2011). Lowering the concentration to the minimum effective limit can also reduce the burden on RNAi machinery and avoid disrupting physiological processes. The causes for the immune response in response to siRNA sequence and length, as well as the siRNA-DDS complexes have been

thoroughly explored in this excellent review: (Jackson and Linsley 2010). For example, it was shown that the motif 5'-UGUGU-3' is immune-stimulatory, and therefore, such a motif should be avoided when considering designing siRNA sequences for targeting bone regeneration (Judge, Sood et al. 2005).

Besides the universal barriers that any siRNA DDS should overcome (Whitehead, Langer et al. 2009), there are a few additional design criteria that need to be considered for bone regeneration applications. In cases of bone fracture or defects, designing an injectable system can be very beneficial for localizing siRNA-loaded particles and maximizing their therapeutic effect. Such an approach will decrease the number of physiological barriers the DDS-siRNA must overcome as compared to systemic administration. Moreover, administering an osteoconductive scaffolding material in conjunction with osteoinductive siRNA-loaded particles can accelerate bone formation and improve bone quality. The addition of targeting moieties is another important criterion needed to improve accuracy of siRNA cellular delivery. The three most commonly used moieties are bisphosphonates, bone-specific aptamers and the poly-aspartic acid sequence. Both the poly-aspartic acid sequence and bisphosphonates target bone mineral rather than a specific cell type, however, the recent study done by Liang et al. directly targets osteoblasts using aptamer-functionalized nanoparticles (Liang, Guo et al. 2015). Finding such moieties that increase specificity to osteoblast lineage will improve the therapeutic effect and clinical relevance of DDS-siRNA systems, to influence bone regeneration at various bone developmental stages. Finally, incorporating low concentrations of osteogenic factors

(e.g. BMPs) along with the osteoinductive siRNA can further improve bone regeneration."

Treatment of large bone defects and particularly CSD is still a challenging orthopaedic procedure which would benefit the most from novel therapeutics, as such, this is certainly an area to be explored in future research. Additionally, it is of great importance to evaluate the pharmacokinetic profile of these delivery systems to safeguard localization of the siRNA and BMP-2 cargo at the desired site of bone regeneration to prevent off-target effects.

Conclusion

Our findings add to the growing body of basic evidence suggesting that Noggin inhibition can enhance the efficacy of BMP-2 on osteoblastogenesis and bone formation. With technological advances in the field of nanotechnology and biomedical engineering, several lipid based nanoparticles are now available. We showed here that the delivery of siRNA to hard-to-transfect osteoblasts is done very efficiently by LNP. The outcomes of this research will undoubtedly assist safe and economical treatment of individuals whose poor bone repair results in permanent morbidity and disability.

siRNAs have emerged as an excellent new genetic tool in biology and are also becoming the next frontier in gene therapy, holding great therapeutic promise. Advanced nanotechnology offers novel solutions for improved and efficient DDS. Nevertheless, our search revealed no current or previous clinical trial utilizing siRNA therapy for musculoskeletal defects or disorders. Investigators have identified a large number of regulatory signals and targets playing central roles in bone development and

repair which offer a long list of potential attractive targets for translational research and siRNA therapeutics. Despite several barriers in the systemic application of such therapeutics, local application of siRNA at the site of bone defects is advantageous and appears to be less complex. High prevalence of skeletal injuries with consequent social, emotional and economic impact on individuals implies significant investment in drug discovery and development in this area and provides a huge unmet global market for pharmaceutical industries.

Future Directions

Additional evaluation, characterization and development of Lipid- Noggin siRNA systems are without doubt necessary and are possible. Also, the experiments could be replicated in different cell types and tested in animal models of bone repair. Our preliminary experiment provided promising evidence that such RNAi approaches have the potential to contribute to the current state of the knowledge in orthopedic reconstructive surgery. For instance, experiments on the collagen 3D scaffolds, which already constitute almost entire common orthopaedic practice for the treatment of large defects, could be expanded to understand physical, chemical and mechanical interactions of such structures within *in vivo* environments. Simultaneously, the LNP-siRNA release profile should be monitored spatially and temporally over the course of bone repair. The use of live *in vivo* imaging devices, such as the one we utilized in the current study, would provide invaluable information in the least invasive manner possible. Perhaps larger animal models of fracture and bone repair would offer more

translational value as their bone repair resembles what occurs in human to a larger scale.

Another essential area to further investigate is the type of LNP to use in vivo, and potentially in human. It is vital to understand whether the LNPs behave the same within the *in vivo* environment as they do *in vitro.*

One area of major attention in modern medicine is targeted therapy. Studies involving special LNPs with moieties specific to damaged bone tissue could answer the question whether successful targeted delivery of Noggin siRNA to the site of bone repair could lead to the development of therapies being administered systemically at small doses during the course of treatment? In this case, the major concern of drug toxicity and diffusion of pharmaceutical agents outside the zone of fracture (to cause adverse events such as ectopic bone formation and unwanted off target effects) could be adequately addressed and this would uncover a wide range of clinical applications using such systemic approaches.

Noggin Is not the only negative regulator of BMP signaling, as such, therapeutics targeting more than one negative regulator, i.e. Germlin and Chordin would lead to the development of even more effective tissue regeneration management techniques. In designing such therapies, L-B-L LNPs and stimulus-responsive injectable systems could offer us with simultaneous or chronological release of various types of growth stimulators during different phases of fracture healing and tissue repair. It is equally important to consider the parallel enhancement of angiogenesis alongside the bone formation to provide sufficient tissue oxygenation and efficient delivery of nutrients and growth factors to the site of newly formed bone.

Future research is necessary in the field of bone substitutes and scaffolds. Larger bone defects would require an appropriate scaffold to provide sufficient 3D mechanical stiffness and resistance, simultaneously, such scaffolds need to be designed specifically to allow the release of therapeutic agents and the growth of new vascular and bone tissue. Advancements in the area of tissue 3D printing, particularly using biological material and cells, will definitely provide us with easier, faster, less expensive and personalized scaffolds for the patients in the future.

Another potential approach would be to target multiple BMP inhibitors (Noggin, Germlin, Chordin) for a more efficient enhancement of BMP signal. Investigating other methods of RNAi, i.e. Antisense Technology (ssDNA) is something to consider as the RNAi technology advances. Recent developments in cell therapy and stem cell research have encouraged advanced autologous cell-based therapies for bone regeneration and treatment of fracture non-unions. As such, development of a simple, safe and efficient strategy to increase the osteoblastic differentiation and bone formation potential of a small number of already available autologous precursor cells would revolutionize this approach.

Of course, before any of these therapeutic strategies can be applied in humans, they need to be tested and it will be crucial to carry out comprehensive experiments to ensure their safety and efficacy.

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6. Appendix

6.1. Journal Publications

REVIEW

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Small Players Ruling the Hard Game: siRNA in Bone Regeneration

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ABSTRACT

Silencing gene expression through a sequence-specific manner can be achieved by small interfering RNAs (siRNAs). The discovery of this process has opened the doors to the development of siRNA therapeutics. Although several preclinical and clinical studies have shown great promise in the treatment of neurological disorders, cancers, dominant disorders, and viral infections with siRNA, siRNA therapy is still gaining ground in musculoskeletal tissue repair and bone regeneration. Here we present a comprehensive review of the literature to summarize different siRNA delivery strategies utilized to enhance bone regeneration. With advancement in understanding the targetable biological pathways involved in bone regeneration and also the rapid progress in siRNA technologies, application of siRNA for bone regeneration has great therapeutic potential. High rates of musculoskeletal injuries and diseases, and their inevitable consequences, impose a huge financial burden on individuals and healthcare systems worldwide. © 2016 American Society for Bone and Mineral Research.

KEY WORDS: SMALL INTERFERING RNA; OLIGOTHERAPEUTIC; GENE THERAPY; DELIVERY SYSTEMS; FRACTURE HEALING; NONUNION BONE HEALING; BONE TISSUE ENGINEERING

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Efficient *in vitro* delivery of Noggin siRNA enhances osteoblastogenesis

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[c] *Department of Biomedical Engineering, McGill University, Montreal, Canada*
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Title:	Small Players Ruling the Hard Game: siRNA in Bone Regeneration
Author:	Saber Ghadakzadeh,Mina Mekhail,Ahmed Aoude,Reggie Hamdy,Maryam Tabrizian
Publication:	Journal of Bone and Mineral Research
Publisher:	John Wiley and Sons
Date:	Mar 8, 2016

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6.3. Resume

SABER GHADAKZADEH, MD., MSc., Ph.D. Candidate

EDUCATION & TRAINING

Ph.D. Experimental Surgery & Biomedical Engineering; McGill, (Montreal, QC) **2014-present**
Supervisors: Dr. Reggie Hamdy and Prof. Maryam Tabrizian
Thesis (*in process):* *"Enhancement of Osteogenesis and Mineralization by Delivery of Noggin siRNA from Lipid-Based Nanoparticles"*
Recipient of several merit-based scholarships and travel awards over 3 years.

M.Sc. Institute of Medical Science, University of Toronto, (Toronto, ON) **2011-2014**
Supervisor: Dr. Benjamin Alman
Thesis: "*Wnt/ β-catenin: A Novel Candidate Pathway for Bone Repair in Neurofibromatosis Type 1".*
Recipient of $130K in merit-based scholarships and awards for 3.5 years.
Secured 100K USD grant: US Department of Defense NF Research Program

M.D. Faculty of Medicine, Jondishapour University, (Ahvaz, Iran) **2000-2008**
Dissertation: "*Evaluation of students and graduates' opinions on the medical ethics course in the medical school curriculum".*
Ranked among top 0.5% in university entrance exam and received of *full scholarship* covering 100% tuition for 7.5 years of medical training (equivalent of over 250K USD).

Other Trainings:

Project Management Foundations, MITACS, (in collaboration with McGill University)**2017**

Project Management: Essential Elements, SKILLSETS, (McGill University) **2017**

Good Clinical Practice, Protecting Human Research Participants, (NIH**,** online) **2016**

Business Skills Training, (McGill University, 1 semester) **2015**

Global Health Diploma, (University of Copenhagen, online) **2013**

RESEARCH, CLINICAL AND MANAGEMENT EXPERIENCE

Shriners Hospital & McGill Biomedical Engineering, (Montreal, QC) **2014-Current**

- **PhD Researcher, Biomat'X Research Laboratories**

Main Projects:

- Review of the literature: utilization of RNAi and siRNA for bone regeneration
- A novel layer-by-layer lipid nanoparticle for orthopedic drug delivery: Design, Generation, Characterization and in vitro functional tests
- siRNA mediated Noggin inhibition to enhance BMP signaling for induced osteoblast differentiation and accelerated mineralization

- *In vivo* Noggin inhabitation (proof of concept): Initiated and designed the experiments, ethical and scientific approval for a new Animal Use Protocol (AUP), animal model of bone repair, microsurgery on mice, *in vivo* imaging

Collaborative projects:

- Sclerostin inhibition through systemic antibody application to augment Wnt signaling and improve distraction osteogenesis
- Postoperative assessment of pedicle screw in spine surgery, development of a novel noninvasive grading tool for spine surgeons

Graduate Student Society, (Montreal, QC) **2015-2016**

- **Vice President of Academic Affairs**

- Planned and coached 5 departmental academic events in 2015, development of 2 new info sessions
- Responded to graduate students' academic inquiries
- Liaised between the students and Experimental Surgery administration, implementing mandatory *Supervisor-Student Memorandum of Agreement* upon acceptance

University Health Network, (Toronto, ON) **2013-2015**

- **Clinical Research Analyst**

- Provided medical, clinical & scientific advice to adult Neurofibromatosis (NF) Clinic
- Established 3 new clinical research registry initiatives and Canadian NF Database, facilitating further research and patient follow up
- Implemented novel data-recording strategies reducing patient waiting times by 60%
- Analyzed NF clinic data to generate 2 publications and 2 international presentations

Hospital for Sick Children, University of Toronto (Toronto, ON) **2010-2014**

- **Graduate Researcher**

- Discovery of impaired β-Catenin signaling pathway regulation in neurofibromatosis type 1 (NF1) bone repair; genetic modulation of Wnt signal in human and mouse stem cells and in NF1 animal models to test novel therapeutic candidates for defective bone regeneration
- Designed state of the art procedures and developed experimental protocols according to the Human and Animal Safety Regulations
- Trial of a patented drug and stem cell therapy to accelerate impaired NF1 fracture repair
- Collaborated with Sanofi to train and coach interns at *BioGENius Challenge Canada*
- Performed project budgeting, selection and purchase of lab equipment and reagents based on SickKids and University of Toronto procurement and regulatory guidelines

Global Medical Brigades, University of Toronto, (Toronto, ON) **2013-2014**

- **Co-President**

- Led the global volunteer medical missions to rural areas in Honduras
- Worked with KOLs to organize biweekly educational and fundraising events for 6 months
- Co-chaired the executive meetings, interviewed 21 individuals and recruited 8 board members

- Built networks with corporate sponsors: Kaplan Medical, Unilever, UT Poster, BiMo, Krispy Kreme
- Arranged an interview with the Minister of Health in Honduras through individual networking to explore future medical and educational collaborations

NF Society of Ontario, (Toronto, ON) **2012-2014**

- **Advisor**

NFSO is a provincial not-for-profit organization focusing on support and information for neurofibromatosis patients and their relatives.
- Acted as a liaison to bridge NF research, NF clinic and NFSO
- Presented NF research and projects for public, raised awareness for NF
- Arranged meetings of the NFSO Board members with the clinic staff

Strategic Planning Core Team, IMS, (Toronto, ON) **2011-2014**

- **Committee Member**

- Prioritized needs and strategized a 5 year Action Plan for IMS
- Represented the community of over 150 international graduates

South Unit Clinic, Emergency Medicine, (Ahvaz, Iran) **2008-2010**

- **General Clinical Practice, GP**

- Led the medical team, supervised the clinic staff including 2 paramedics and 2 nurses
- Provided outpatient/inpatient care for a population of 1600 individuals in remote areas

PUBLICATIONS

Postoperative Assessment of Pedicle Screw and Management of Breach: A Survey among Canadian Spine Surgeons and a New Scoring System
S. Ghadakzadeh*, Ahmed Aoude*, Hamzah Alhamzah, Maryse Fortin,Peter Jarzem, Jean Ouellet, Michael H. Weber, Asian Spine Journal 2018 Feb;12(1):37-46. English. doi.org/10.4184/asj.2018.12.1.37 **2018**

Efficient in vitro delivery of Noggin siRNA enhances osteoblastogenesis
S. Ghadakzadeh, R. Hamdy, M. Tabrizian, Bone Reports, Heliyon Nov; (3) e00450. **2017**

Assessment of the effect of systemic delivery of sclerostin antibodies on Wnt signaling in distraction osteogenesis
Mohammad M. Alzahrani, Asim M. Makhdom, Frank Rauch, Dominique Lauzier, Maria Kotsiopriftis, **S. Ghadakzadeh**, R. Hamdy, Bone and Mineral Research, DOI: 10.1007/s00774-017-0847-2. **2017**

One-year report on the structure and clinical volume of 1st Canadian adult NF1 multidisciplinary clinic
Alireza Mansouri*, **Saber Ghadakzadeh***,Talha Maqbool, Carolina Barnett, Karolyn Au, Paul Kongkham, Vera Brill, Gelareh Zadeh, Canadian Journal of Neurological Science, Sep; 44(5):577-588. **2016**

β-Catenin modulation in neurofibromatosis type 1 bone repair: therapeutic implications
S. Ghadakzadeh, Peter Kannu, Heather Whetstone, Andrew Howard, Benjamin A Alman FASEB Journal, 2016 Sep;30(9):3227-37. **2016**

Small Players Ruling the Hard Game: siRNA in Bone Regeneration
Saber Ghadakzadeh, Mina Mekhail, Ahmed Aoude, Maryam Tabrizian, Reggie C Hamdy, Journal of Bone and Mineral Research (JBMR), Jul;31(7):1481, **2016**

siRNA-mediated Noggin inhibition enhances osteogenesis and mineralization
Saber Ghadakzadeh, Mina Mekhail, Reggie Hamdy, Maryam Tabrizian, Bone Abstracts, DOI:10.1530/boneabs.5.LB15, **2016**

Body Image Concern Inventory (BICI) for Identifying Patients with BDD Seeking Rhinoplasty: Using a Persian (Farsi) Version
Saber Ghadakzadeh, Ali Ghazipour, Niloufar Khajeddin, Negar Karimian, Mehrdad Borhani, Aesthetic Plastic Surgery 04/2011; 35(6):989-94., DOI:10.1007/s00266-011-9718-8, **2011**

Swyer syndrome in a woman with pure 46,XY gonadal dysgenesis and a hypoplastic uterus: A rare presentation
Negar Karimian, **Saber Ghadakzadeh**, Mahdi Eshraghi, Fertility and sterility 11/2009; 93(1):267.e13-4., DOI:10.1016/j.fertnstert.2009.09.062, **2009**

Self-inflicted non-healing genital ulcer: a rare form of factitious disorder
A Feily, M R Namazi, M Saboktakin, M Mehri, J Lotfi, A Ayoobi, **S Ghadakzadeh**, N Karimian, Acta dermatovenerologica Alpina, Panonica, et Adriatica 07/2009; 18(2):83-5. **2009**

Evaluation of medical students and graduates' opinions on the medical ethics course in the medical school curriculum in 2006-2007
Saber Ghadakzadeh, Saadati N, Dibaei A, Medical Ethics, 2009, 3(7): 111-139. **2009**

PRESENTATIONS AND CONFERENCE PROCEEDINGS

siRNA-mediated noggin inhibition enhances Osteogenesis
Saber Ghadakzadeh, M Mekhail, R C Hamdy, M Tabrizian, Faculty of Dentistry Research Day, Montreal, QC. **2016**

Enhancement of Osteogenesis by Lipid-Based Delivery of Noggin siRNA
Saber Ghadakzadeh, M Mekhail, R C Hamdy, M Tabrizian, Journée scientifique du RSBO,

Montreal, QC, **2016**

Improving Bone Healing in Neurofibromatosis: A Study in Mice
Benjamin Alman, **Saber Ghadakzadeh**, Heather Whetstone, Gurpreet Baht, American Academy of Orthopaedic Surgeons, Annual Meeting, Orlando, Florida, **2016**

One-year institutional report of the first Canadian adult NF1 multidisciplinary clinic
Alireza Mansouri*, **Saber Ghadakzadeh***,Talha Maqbool, Carolina Barnett, Karolyn Au, Paul Kongkham, Vera Brill, Gelareh Zadeh, 20th Annual Scientific Meeting of the Society for Neuro-Oncology, San Antonio, United States, **2015**

A Lipid-based Nano-delivery System of Noggin siRNA to Enhance Bone Regeneration
Saber Ghadakzadeh, Mina Mekhail, Maryam Tabrizian, Reggie C Hamdy, 11th Annual Meeting of the Oligonucleotide Therapeutics Society, Leiden, Netherlands, **2015**

Wnt/[beta]-catenin: a candidate pathway for bone repair in neurofibromatosis type-1
Ghadakzadeh Saber, Amini Nik Saeid, Baht Gurpreet, Whetstone Heather, Alman Benjamin, Bone Abstracts, International Conference on Children's Bone Health
DOI:10.1530/boneabs.2.OC25, **2013**

Wnt/ ß-catenin: A candidate pathway for bone repair in Neurofibromatosis Type-1
Ghadakzadeh S, Baht G, Whetstone H and Alman B. ICCBH abstract Book. International Conference on Children's Bone Health, Rotterdam, Netherlands. **2013**

ß -Catenin Activation Inhibits Bone Repair in Neurofibromatosis: Implications for A Novel Approach to Improve Bone Healing
Ghadakzadeh S, Whetstone H and Alman B. (2013). 39th Gallie-Bateman & McMurrich research Presentations. Toronto, Canada. **2013**

Improving Bone Healing in Neurofibromatosis: A Study in Mice
Ghadakzadeh S, Wetstone H and Alman B. POSNA Scientific Program. The Pediatric Orthopaedic Society of North America - POSNA, Toronto, Canada. **2013**

Bone Healing in NF1 Is Through Wnt/ β-catenin Pathway
Ghadakzadeh S, Nik S, Baht G, Whetstone H and Alman B. Musculoskeletal Biology & Bioengineering, Gordon Research Conference, Proctor, NH, USA. **2012**

Wnt/ β-Catenin Pathway Mediates Bone Repair in Neurofibromatosis Type-1
Ghadakzadeh S and Alman B. IMS Scientific Day abstract book. Toronto, Canada. **2012**

Wnt/ β-Catenin Pathway Mediates Bone Repair in Neurofibromatosis Type-1
Ghadakzadeh S and Alman B. 38th Annual Gallie-Bateman & McMurrich research Presentations, **2012**

Beauty concepts & body dysmorphic disorders; Presenting Body Image Concern Inventory (BICI) for Identifying BDD Patients Seeking Rhinoplasty: Using a Persian (Farsi) Version

Ghadakzadeh S, Ghazipour A, Khajeddin N, Karimian N, Borhani M. Kosmetische Medizin. Cosmoderm XVI, The European Society for Cosmetic & Aesthetic Dermatology (ESCAD), Dresden, Germany. **2010**