Preclinical Assessment of Novel Strategies to Enhance Bone Regeneration

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Abstract

Skeletal reconstruction is a common challenge faced by orthopedic, dental, and maxillofacial surgeons. Although autogenous bone graft, which is the gold standard of grafting material, has excellent mechanical and biological properties, its scarcity and associated donor site morbidities limit its application in clinical settings. Allogeneic and xenogeneic bone grafts are advantageous due to their availability, but their use is complicated by their inferior osteogenic capacity and risk of transmitting infections. Synthetic bone grafts consisting of scaffold(s), cells, and/or growth factor(s) have emerged as a promising substitute for natural bone grafts for skeletal reconstruction. Consistent with the notion that osteogenesis and angiogenesis are coupled processes during embryogenic bone formation and postnatal bone healing, this thesis suggests that the transplantation of bone marrow derived osteoprogenitor cells (mesenchymal stromal/stem cells [MSC]) in combination with the delivery of angiogenic growth factor (vascular endothelial growth factor [VEGF]) can be utilized to improve bone regeneration.

MSC were isolated from the bone marrow of 4-month-old C3H WT mice and expanded *in vitro* before being transplanted into recipient mice. The MSC transplantation alone was able to improve peri-implant bone regeneration and fixation of an implant in poor quality bone as shown in a mouse model of femoral implant osseointegration simulating human hip prostheses (Chapter III). A novel murine model was developed to investigate the reconstruction of large-sized defects, such as those left after tumor resection or arising from major traumatic injuries. The MSC that were seeded in a biomimetic collagen scaffold *in vitro* and transplanted into the large defect were unable to promote sufficient bone regeneration to restore skeletal integrity. When supplemented with a bolus dose of VEGF treatment, the MSC-seeded dense collagen scaffold exhibited a higher degree of vascularization and integration with the recipient bone, which

accounted for the significantly greater bone formation in the defect and the promotion of defect healing (Chapter IV). In addition to the bolus injection, VEGF covalently tethered with a collagen sponge was able to increase angiogenesis and bone healing of a critical-sized rat mandibular defect as a model of reconstruction of a critical-sized bone defect in the maxillofacial area (Chapter V).

MSC transplantation is a promising approach to improve the osseointegration of implants used in bone reconstruction. A tissue engineering approach (combining a collagen biomimetic scaffold, MSC and VEGF) is able to promote the repair of large-sized bone defects. Thus, preclinical studies using large animal models and clinical trials are warranted.

Résumé

La reconstruction squelettique représente un défi majeur auquel sont confrontés les chirurgiens orthopédiques, dentaires et maxillo-faciaux. L'autogreffe osseuse est la méthode standard en or en raison de ses hautes propriétés mécaniques et biologiques. Cependant, son utilisation est limitée en raison de sa rareté et de sa morbidité associée au site donneur, conduisant ainsi à une limitation de son application en milieux cliniques. Les greffes osseuses allogéniques et xénogéniques, quant à elles, sont nettement avantageuses pour leurs disponibilités. Cependant, leur utilisation est confrontée à certaines difficultés, notamment à ce qui attrait à leur faible capacité ostéogénique, et au risque élevé de contamination. Toutefois, avec les avancées réalisées en Ingénierie tissulaire, les greffes osseuses synthétiques comprenant, les échafauds, les cellules et /ou les facteurs de croissance ont émergées en tant que substituts prometteurs pour les greffes d'os naturels ciblant la reconstruction squelettique. Conformément à l'idée que l'ostéogénèse et l'angiogénèse sont deux processus couplés lors de la formation de l'os embryonnaire et de la cicatrisation osseuse postnatale, mes travaux de recherche ont pour objectifs d'étudier l'effet combiné de la transplantation des cellules souches mésenchymateuses stromales (MSC), dérivés de la moelle osseuse, avec la livraison du facteur de croissance angiogénique (VEGF), en vu d'une amélioration de la régénération osseuse.

Les MSC sont isolées de la moelle osseuse de souris C3H sauvages âgées de 4 mois, et cultivées *in-vitro* avant leur transplantation chez des souris receveuses. La transplantation des MSC a pu améliorer la régénération osseuse péri-implantaire ainsi que la fixation de l'implant dans un os qualifié de mauvaise qualité, ces résultats concordent avec ceux d'une étude réalisée chez des souris ayant reçues un implant au niveau de la hanche visant à stimuler l'osséointégration (Chapitre III).

Un nouveau modèle murin a été développé afin d'investiguer la reconstruction osseuse au niveau d'un défaut osseux critique, comme ceux résultant d'une résection tumorale ou découlant de traumatismes. Les MSC incorporées dans un échafaudage de collagène ex-vivo et transplanté dans un défaut osseux étaient incapables de favoriser la régénération osseuse pour rétablir l'intégrité du squelette. Cependant, leur injection conjointe avec un bolus de VEGF a montré que les MSC entaient capables d'induire un haut niveau de vascularisation et d'intégration avec l'os receveur, ce qui a conduit à une augmentation de la formation osseuse au niveau du défaut osseux mais aussi promouvoit la reconstruction osseuse. De plus, la liaison du VEGF à l'éponge du collagène a pu augmenter l'angiogénèse et la reconstruction osseuse chez un modèle de rat avec un défaut mandibulaire de taille critique, utilisé comme modèle mimant un défaut osseux généré au niveau de la zone maxillo-faciale (chapitre V).

La transplantation des MSC représente une approche prometteuse pour améliorer l'ostéointégration des implants utilisés dans la reconstruction osseuse. Une approche de l'ingénierie tissulaire combinant un échafaudage de collagène biomimétique, MSC et VEGF est capable de promouvoir des défauts osseux importants. Ainsi, des études précliniques sur des modèles animaux grands et des essais cliniques sont nécessaires.

Acknowledgements

Foremost, I would like to express my sincere gratitude to my inspiring and encouraging PhD supervisors, Drs. Janet Henderson and Edward Harvey, for their continuous guidance and generous support for my PhD study. Dr. Henderson's lab staff and summer students have lent large volume of technical assistance to me which was indispensable for completion of my research work. Her collaborators, including Dr. Lisbet Haglund, Dr. Jan Seuntjens, Dr. Michael D. Buschmann, Dr. Rubens Albuquerque and McGill Institute for Advanced Materials (MIAM), provided the materials, facilities and technical assistance required in my experiments. I hereby wish to thank my academic advisor, Dr. Srikant Coimbatore, and my thesis committee members, Drs. Suzanne Morin, Robert Turcotte, Jan Seuntjens, and Dominique Shum-Tim, for their constructive comments the progression of my research over these years. I also like to acknowledge my cousin, Yicong Liu, for her contributions in statistical data analysis, Dr. Kenneth Finnson for his editorial help for my thesis and Dr. Nadia Zayed for the French translation of my abstract.

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Finally and most importantly, thank to my parents and my beloved one. Their love and encouragements energized me to complete the research work and succeed in pursing my PhD degree.

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Abbreviations

ACP: amorphous calcium phosphate

ADSC: adipose derived stem cell

ALIF: anterior lumbar interbody fusion

ALP: alkaline phosphatase

BCP: biphasic calcium phosphate

BG: bioactive glasses

BMA: bone marrow aspiration

BMD: bone mineral density

BM-EPC: bone marrow derived endothelial progenitor cell

BM-MSC: bone marrow derived mesenchymal stem cell

BMP: bone morphogenetic protein

BTE: bone tissue engineering

BV/TV: bone volume per tissue volume

CAD: Computer-aided Design

CCR: C-C chemokine receptor

CHA: carbonated hydroxyapatite

CXCR: C-X-C chemokine receptor

DA: degree of anisotropy

DBM: demineralized bone matrix

DMEM: Dulbecco's Modified Eagle's Medium

EC: endothelial cell

ECM: extracellular matrix

EDC: 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide

EDTA: ethylenediaminetetraacetic acid

EGF: epidermal growth factor

EMEA: European Medicines Agency

EPC: endothelial progenitor cell

ESC: embryonic stem cell

FACS: fluorescence-activated cell sorting

FBS: fetal bovine serum

FDA: US Food and Drug Administration

FGF: fibroblast growth factor

FGFR: fibroblast growth factor receptor

GAPDH: glyceraldehyde 3-phosphate dehydrogenase

GDF: growth differentiation factor

GFP: green fluorescent protein

HA: hyaluronan

HA-TCP: hydroxyapatite-tricalcium phosphate

HBV: hepatitis B virus

HIF1α: hypoxia-inducible factor 1-alpha

HIV: human immunodeficiency virus

HLA: human leukocyte antigen

HLA-DR: human leukocyte antigen-DR

HSC: hematopoietic stem cell

HUVEC: human umbilical vein endothelial cell

Ihh: Indian hedgehog homolog

IL: interleukin

iPC: induced pluripotent stem cell

ISKD: Intramedullary Skeletal Kinetic Distractor

MAPK: mitogen-activated protein kinase

MCSF: macrophage colony-stimulating factor

MEM: minimum essential media

microCT: micro computer tomography

MMP: matrix metalloproteinase

MRF: myogenic regulatory factor

MSC: mesenchymal stem cell

MYF: myogenic factor

NHS: N-hydroxysuccinimide

NRP: neuropilin

NSAID: non-steroidal anti-inflammatory drug

OCN: osteocalcin

OI: osteogenesis imperfecta

OPG: osteoprotegerin

PB-EPC: peripheral blood endothelial progenitor cell

PBS: phosphate buffered saline

PCL: poly(3-caprolactone)

PCR: polymerase chain reaction

PDGF: platelet-derived growth factor

PDS: polydioxanone

PF: paraformaldehyde

PGA: polyglycolide

PHEMA: poly(2-hydroxyethyl methacrylate)

PLA: polylactide

PLGA: poly(lactic-co-glycolic acid)

PMMA: polymethylmethacrylate

PPAR: peroxisome proliferator-activated receptor

PRP: platelet rich plasma

PTH1R: parathyroid hormone 1 receptor

PTHrP: parathyroid hormone-related protein

qPCR: quantitative polymerase chain reaction

RANKL: receptor activator of nuclear factor kappa-B ligand

RGD: arginine-glycine-aspartic acid

rhBMP: recombinant human bone morphogenetic protein

rhFGF: recombinant human fibroblast growth factor

rhPDGF: recombinant human platelet derived growth factor

rhVEGF: recombinant human vascular endothelial growth factor

RIA: Reamer Irrigator System

ROI: region of interest

RT-PCR: reverse transcription polymerase chain reaction

RUNX: runt-related transcription factor

SAM: self-assembled monolayer

SFF: Solid Free Fabrication

SMI: structure model index

SVF: stromal vascular fraction

Tb.N: trabecular number

Tb.Pf: trabecular pattern factor

Tb.Sp: trabecular separation

Tb.Th: trabecular thickness

TCP: tricalcium phosphate

TCP: tissue culture plastic

TGF: transforming growth factor

Ti: titanium

TIE: tyrosine kinase with immunoglobulin-like and EGF-like domains

TNF: tumor necrosis factor

TRAP: tartrate resistant acid phosphatase

UCSC: umbilical cord stromal cell

UHMWPE: ultrahigh molecular weight polyethylene

VBG: vascularized bone graft

VEGF: vascular endothelial growth factor

VEGFR: vascular endothelial growth factor receptor

VOI: volume of interest

Contributions

In accordance with the Thesis Guidelines stipulated by McGill Graduate and Postdoctoral Studies, I elected to present my thesis in a manuscript-based format fulfilling the criteria. Chapter I is an overview of the theme of my PhD thesis and Chapter II is a comprehensive literature review of the background and context in which my PhD research was initiated. My original intellectual contributions are presented in Chapter III, IV and V; written in the form of original papers, encompassing their respective abstract, introduction, materials and methods, results, discussion and conclusion-integrated by connecting preface. The manuscripts included in Chapters III and IV are published in peer review journals and the manuscript in Chapter V is in preparation for submission. Chapter VI is a discussion about the results obtained from my PhD work, current issues to be addressed in the same research field and future directions in the field.

As the first author of the manuscripts included in this thesis, I established the experimental protocols, conducted the bulk of the experiments, and performed data collection and analysis as well as paper drafting. Drs. Janet Henderson and Edward Harvey provided essential supervision throughout the project and edited the manuscripts for publishing. As a collaborator, Dr. Jan Seuntjens helped with irradiation conditioning of murine recipients for stem cell transplantation. Gabriel N. Kaufman did the growth and differentiation assay shown in Chapter III. Several other undergraduates and medical students, including Mardon Chua, Alison Butler, Brian Chen, and Fan Jiang, contribute to my work by assisting in cell culture, qPCR, micro computed tomography (microCT) scanning and histological processing under my supervision. Yicong Liu verified the validity of statistical analysis shown in published manuscript included in Chapter IV. My peer graduate students, Zaher Jabbour and Saad

AlQahtani, helped me in the animal surgery and manuscript preparation presented in Chapter V. Ailian Li and Huifen Wang, our lab's research assistants, provided technical assistance in histological analysis and animal maintenance, respectively. The contributors appear on the authorship or acknowledgement of the manuscripts to reflect their involvement in experimental or analytical aspect(s) of the presented works.

In addition to the manuscripts presented in Chapters III, IV and V, I contributed to several other studies as a co-author as shown in Appendix III. My contribution included establishing rodent models, performing quantitative microCT analysis, histopathological data interpretation and manuscript preparation. Appendix I is a description of the methodologies essential to this PhD thesis work and collaborative projects and includes some unpublished data I generated. Appendix II lists the publications that I contributed to as a co-author.

Contributions to original knowledge

Bone tissue engineering has seen tremendous progress in the past decade with cell-assisted strategies emerging as a promising approach to bone augmentation. However, we still lack convincing data on the therapeutic efficacy of transplanting MSC in enhancing bone regeneration, especially when the endogenous bone healing is impaired.

The work in Chapter III has for the first time shown that the transplantation of allogeneic MSC can improve peri-implant bone regeneration and osseointegration of Ti implant in recipients that are characterized by reduced availability of osteoprogenitor cells.

Chapter IV describes the work that evaluated the potential of allogeneic MSC transplanted via dense collagen scaffolds in expediting healing of large bone defects. Extensive *in vitro* studies of the dense collagen gel have been done by previous authors to evaluate its potential as a novel synthetic bone graft; however, the current work is the first one that assessed the dense collagen scaffold *in vivo* in a clinically relevant orthotopic bone healing model. This large-sized bone defect murine model was originally developed in this PhD work and proved to be a valid model for preclinical study of bone tissue engineering. It was found that a bolus dose of VEGF treatment was able to promote angiogenesis and function synergistically with the allogeneic MSC-seeded dense collagen scaffold to expedite the healing of large bone defects with significantly improved integration of the transplanted scaffold. Either of these treatments alone failed to induce a comparable therapeutic effect with this model.

As an extension of the work described in Chapter V, the therapeutic potential of VEGF in healing critical-sized bone defect was further evaluated in a rat mandible defect model. Instead of administrating VEGF as a bolus injection, VEGF covalently tethered to collagen sponge by

using EDC chemistry was applied as a therapeutic in this project. It was shown for the first time that VEGF, when covalently tethered with collagen sponge, was able to enhance vascularization and expedite the healing of a critical-sized bone defect.

Other original contributions made during my PhD program include the initial preclinical evaluation of Ti foam developed by National Research Council (NRC) in a critical-sized segmental defect model of rat femur and the development of a fracture nonunion model of minipig scaphoid. The unpublished data that I generated is included in the Appendix I.

Overall, allogeneic MSC transplantation seems to serve as a viable therapeutic strategy to augment bone formation and improves the osseointegration of intraosseous implants. In the context of large-sized bone defects, VEGF treatment plays a role in inducing angiogenesis and functions synergistically with transplanted MSC to enable robust *en bloc* bone formation and expedited bone healing.

Chapter I: Introduction

Bone defects or loss of skeletal structural integrity can be caused by pathological conditions, including infection, fracture non-union, large-scaled traumatic injury, congenital and developmental deformities, as well as invasive procedures such as tooth extraction and revision surgeries for bone tumor, osteomyelitis, and degenerative joint disease [1-4]. As the baby boomers enter their 60s and 70s in the coming decade, osteoporosis and degenerative joint disease will become increasingly more prevalent health issues for society, since an ageing population is at higher risk of skeletal traumatic injury, delayed union or non-union, and complex joint replacement and revision surgeries [5 6 7]. In these commonly encountered clinical scenarios, the spontaneous healing of bone defects is frequently disrupted, and if left untreated will inevitably cause an impairment of function, a lack of stability of implant in bone, and a poor aesthetic outcome [8]. As such, the reconstruction of skeletal structural integrity is a common challenge shared by orthopedic, dental, and craniofacial surgeons.

Bone grafts were first used in skeletal reconstruction in the 17th century. For example, Christian church literature recorded the transplantation of a dog's cranial bone into a Russian soldier in 1682 [9]. After centuries of evolution, bone grafting has become a common procedure for bone augmentation, and grafting techniques have been refined to minimize morbidity. Natural bone grafts can be autogenous, allogeneic, and xenogenous [10]. Imbued with excellent osteogenic capacity and deficient in immune reactions, autogenous bone grafting is superior to allograft and xenograft, and is currently recognized as the gold standard for grafting procedures [11 10]. However, the scarcity of the resource and its inherent self-resorption limit the potential of the autograft for bone defect reconstruction, especially those of critical-size [12]. Over the past 50 years, enormous progress has been made in surgical techniques and devices used to reconstruct

skeletal defects, among which are internal bone transports that use the principle of distraction osteogenesis [13] [14], intramedullary lengthening [15], and bioactive pseudomembrane techniques including Masquelet technique [16] and cylindrical mesh technique [17]. The combinations of these smart designs and sophisticated operations have enabled *en bloc* bone formation and also have provided plausible approaches for repairing large-scaled bone defects in extremely challenging cases. However, these approaches require numerous staged operations, long-termed fixation, and lengthy hospital stays that are frequently complicated with a higher risk of infection and joint contracture [12].

Synthetic or engineered bone substitutes that combine biomaterials, osteogenic cells, and/or osteobiologics have shown a potential to be the ultimate solution for the reconstruction of bone defects. Significant progress in biomaterial science, cell biology, and developmental biology have made it possible to manufacture customized equivalents to bone grafts with optimal physical, chemical, and biological characteristics so that these customized grafts can be manufactured specifically for the particular bone defect that needs repair, provide sufficient mechanical support, integrate into the recipient bones, and facilitate *de novo* bone formation *in vivo* [18] [19] [20]. With unlimited availability, these kinds of engineered bone grafts can theoretically produce outcomes similar to autogenous bone grafts, the bone graft gold standard, plus they can avoid the shortcomings inherent to natural bone grafts [21]. However, clinically applicable bone engineering therapeutics for enhancing bone regeneration are still lacking, especially with respect to poor-quality recipient bone, although the significant advances achieved in related basic science fields have been encouraging.

Aiming to explore novel bone engineering therapeutics that can be readily translated into clinical practice, the present study focuses on an evaluation of potential osteogenic therapeutics prepared in the lab by using pre-clinical rodent models with clinical relevance.

Chapter II: Literature Review

2.1. Skeletal Healing

Bone healing is a complex biological process with several overlapping stages involving numerous cellular and molecular events that are tightly coordinated. Studies have shown that bone regeneration after injury recapitulates the embryogenic bone formation that enables bone to heal with tissue indistinguishable from uninjured tissue [22]. Thus, common cellular players and molecular control mechanisms underlie the osteogenesis of both skeletal development and repair [23]. Apparently, bone formation is achieved by the cooperation and interactions among various types of cells, such as mesenchymal stem cells (MSC), chondrocytes, osteoblasts, and osteoclasts [24], which are coordinated by the crucial molecules categorized as cytokines, growth factor, hormone, extracellular matrix, and others [22 25 26]. At the molecular level, four regulatory mechanisms are paramount to inducing bone formation [23]: 1) the Ihh-PTHrP axis— Ihh and PTHrP are needed for chondrocytic proliferation, hypertrophy, and osteoblast differentiation [27], 2) bone morphogenetic proteins (BMPs)—BMPs are competent inducers of mesenchymal condensation and osteoblast differentiation, and thus play pivotal roles in the initiation, progression, and maturation of osteogenesis [28], 3) Wnt-β-catenin pathway—Wnt signaling pathways stimulate either the proliferation or differentiation of MSC toward an osteoblastic lineage according to the variations in its local concentrations, and it inhibits the formation of chondrocytes and adipocytes [29], 4) Mitogen-activated protein kinases (MAPK)—the MAPK pathway, as the connector between the extracellular environment and osteoblastic differentiation, is the signal transduction mechanism downstream of adhesion molecules (e.g., integrins), several growth factors (e.g., the epidermal growth factor receptor and the fibroblast growth factor

receptor), and inflammatory cytokines (e.g., IL-1, IL-6, and TNF-α) [30]. The MAPK pathway regulates osteoblastic differentiation by interacting with other osteoblastic signaling pathways discussed previously via phosphorylating their key intracellular signal transduction molecules [23, 31].

Nevertheless, the skeletal post-natal healing process is distinguishable from embryonic osteogenesis in several aspects [32]: 1) the presence of inflammation after injury, 2) the relative scarcity of pluripotent stem or osteogenitor cells, and 3) the presence of mechanical forces. Following skeletal injury, osteogenic signals are released from various types of surrounding cells and the degraded extracellular matrix, which, in turn, trigger the establishment of a morphogenic field around the injury site by the cellular components of bone marrow, cortical bone, periosteum, and external soft tissues [33]. Generally speaking, three groups of soluble signaling factors govern the whole bone healing process: 1) pro-inflammatory cytokines—IL-1, IL-6, TNF-α, MCSF, RANKL, and others; 2) TGF-β superfamily—TGFs, BMPs, and GDFs, 3) angiogenic factors—VEGFs and angiopoietins [32]. Due to the differences in the anatomical and mechanical properties of skeletal injuries, different healing mechanisms are employed during the repair process.

Similar to many other tissues, bone can self-repair both by primary and secondary healing [33]. Primary healing is characterized by minimal callus formation and the direct bridging of fracture ends [34], whereas secondary bone healing displays abundant bone callus around the fracture site and a lengthy remodelling process [22]. Unlike the secondary healing of soft tissues, bone healing is capable of generating bone tissue with almost the same mechanical and anatomical properties as those of the original bone [22].

2.1.1. Primary healing

The primary healing mechanism is permitted for conditions in which a precise anatomical structure is restored, and fracture segments are rigidly stabilized [35] [33]. In these circumstances, bone callus is absent, and bone union is achieved by the formation of multicellular remodeling units known as "cutting cones," which is an attempt of bone structure to directly re-establish itself [34]. The cytokine cascade following injury involves upregulated IL-1, IL-6, and RANKL and downregulated OPG [36] that elicit the resorptive reactions of osteoclasts to give birth to the "tunneling" within bone structure, which is used by penetrating blood vessels at a later stage [36] [37]. These blood vessels are composed of both endothelial cells and perivascular mesenchymal cells, one of the osteoprogenitor cells. Thus, osteoblasts at the trailing margin of the multicellular remodeling unit are known to lay down lamellar bone with re-established harversian systems, which serves as a vasculature network [37]. This healing mechanism also underlies the repair process of stress fracture and some fracture cases that undergo anatomical reduction and rigid internal fixation [37].

2.1.2. Secondary healing

As the dominant bone healing pattern in the vast majority of circumstances, secondary skeletal healing is characterized by the formation of bone callus that reaches beyond the limit of the fracture site per se [32]. Two different mechanisms—intramembranous and endochondral ossifications—underlie bone regeneration in the secondary healing process [22]. With respect to intramembranous ossification, the osteogenic cells located in the periosteum directly lay down bone matrix and form hard bone callus when stimulated by the osteoinductive signals released by the bone injury [38]. In contrast, bone regeneration through the endochondral ossification process,

which is comparable to the embryonic osteogenesis of long bones and vertebrae, starts with the condensation of stromal cells to form a preliminary cartilage template that later undergoes degradation, vascularization, and replacement by bone tissue [39] [32]. However, the temporary cartilage template at the fracture site displays more cell components than embryonic bone development and performs its distinct function as a stabilizer of the fragments. Based on the physiological functions of the cells present in the fracture site and signal molecules expressed, the fracture healing process can be divided into four stages (Fig 2.1) [40].

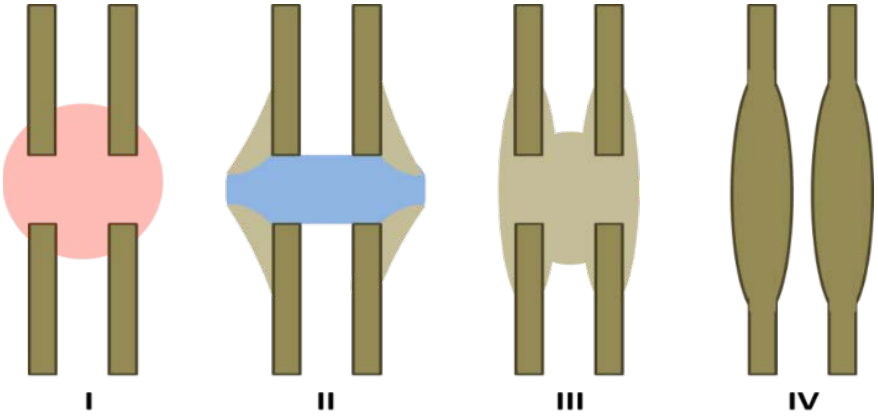

Figure 2.1. Physiological facture healing process. Stage I is characterized by the formation of hematoma with an activation of the inflammatory system and a coagulation cascade. Stage II is dominated by a periosteum reaction and temporary cartilage template formation at the fracture site. In Stage III, the temporary cartilage template undergoes mineralization and replacement by bone callus. In Stage IV, the bone callus is remodeled into a cortical bone structure, and the bone marrow cavity is re-cannulated.

2.1.2.1. Inflammation

Within the first few days following a fracture, a coagulation cascade and an inflammation response are the predominant biological events at the injury location. At this stage, the pro-inflammatory cytokines that are produced play a pivotal role in initiating the healing process [41] [42]. Platelets release certain growth factors such as TGF-β and PDGF after undergoing adherence, degranulation, and activation [43]. Later, inflammatory cytokines (including IL-1, IL-6, RANKL, MCSF, and TNF-α) are produced by the local inflammatory and immune cells, i.e., neutrophils, monocytes, and macrophages. They aid the chemotaxis of mesenchymal stem cells and stimulate their propagation [44]. The enhanced proliferation of MSC induced by PDGF and TNF-α has been shown to be related with the MAPK intracellular signalling pathway [23] [45]. BMP2 has been shown to be expressed at the maximum level within the first 24 hours after fracture and is believed to contribute significantly to the initiation of the healing cascade and the regulation of other BMPs of the TGF-β superfamily [46] [47]. GDF-8, another member of TGF-β superfamily, has been reported to be expressed only on day 1, and its role in controlling cellular proliferation has been suggested [48].

2.1.2.2. Cartilage formation and periosteal response

During the first week after fracture, hard bone callus is formed by the periosteum and internal soft tissues by means of an intramembranous ossification process, which is the same osteogenic mechanism seen in the embryogenic development of the cranium and facial bones [49]. This intramembranous callus formation is initiated from the proximal and distal ends of the periosteal response and evolves into a crescent shaped morphogenic field region. In addition to the osteogenesis that occurs at this stage is the angiogenesis that is needed for efficient mass

transportation and for supplementing the callus with osteoprogenitor cells [50]. Pericytes, which are part of the blood vessel structure, are recognized as one type of osteogenic cell that differentiates into osteoblasts and contributes to bone formation during the postnatal bone healing process [51]. Several BMPs (e.g., BMP2, BMP4, BMP5, and BMP6) are expressed at a high level at this stage and are believed to stimulate the surrounding stem cells into osteoblastic differentiation [52]; meanwhile, the angiogenesis is dictated by the synergistic effect of VEGF and angiopoietin [44].

While the hard callus formed by intramembranous ossification reaches beyond the boundaries of the fracture, a soft cartilaginous callus rich in Type II collagen and aggrecan is formed both within the bone marrow to bridge the fracture fragments and outside the bone cortices to overlie the fracture site [33]. This process is driven by the highly expressed TGF-βs (TGF-β2 and TGF-β3) so that the injury site is provided with a template for the temporary fulfillment of mechanical function and later bony healing [53] [52]. Similar to the growth events in the epiphyseal plate of long bones, the growth of cartilage callus results from the combined contribution of chondrocytic proliferation, hypertrophy, and extracellular matrix production. However, the cartilage callus displays more cellular components and less extracellular matrix when compared to the epiphyseal plate, which some researchers suggest contains a property that facilitates the quick growth of callus that is required by bone healing [32].

2.1.2.3. Cartilage resorption and primary bone formation

The proliferative activities of osteoblasts disappear in the intramembranous process 2–3 weeks after fracture when a substantial amount of woven bone already has been formed adjacent to the fracture site [33]. Later, at approximately 3–4 weeks after fracture, the chondrocytic proliferation in the callus vanishes, and the cartilaginous callus starts to be replaced by primary

spongy bone. Declining cellular proliferation is accompanied by chondrocytic hypertrophy [48] [32]. Hypertrophic chondrocytes become the dominant cell type in the chondroid callus and are the key regulators for the subsequent endochondral ossification process as in the growth plate [54] [33]. Within the cytoplasm of the hypertrophic chondrocytes are vesicularized bodies derived from the buddings of membrane structure, known as matrix vesicles, which transport calcium from the mitochondria to the extracellular matrix and release enzymes, such as phosphatase and protease (e.g., various specific metalloproteinases). These enzymes degrade large matrix proteins and proteoglycans, as well as other anti-mineralization molecules so to mediate the calcification and disintegration of the cartilage matrix [55] [56]. From a morphological perspective, the calcified cartilage in the fracture callus is similar to the primary spongiosa in the growth plate. Thereafter, hypertrophic chondrocytes play an important role in inducing the ingrowth of the penetrating blood vessels by releasing VEGF, a chemotactic and mitogenic factor [57] [58]. The newly formed vasculature serves as the channel that chondroclasts and osteoprogenitors utilize to migrate into the calcified cartilage under the chemotactic effects exerted by the upregulated certain cytokines (e.g., RANKL, OPG and MSCF) and BMPs (BMP2-8), respectively [44] [32]. These locally distributed signal molecules also induce the differentiation and functional maturation of chondroclasts and osteoblasts [44] [32]. As a result, the calcified matrix is resorbed and thus provides a scaffold for osteoblasts to attach and make the woven bone that is similar to the secondary spongiosa of the growth plate. Unlike IL-1 and IL-6, TNF-α also is upregulated at this stage and reported to contribute to the absorption of the calcified matrix and induce the apoptosis of hypertrophic chondrocytes [59].

2.1.2.4. Secondary bone formation and remodeling

During this phase, bone marrow is reconstituted, and cortex is built with lamellar bone and Haversian systems. This process can extend 12 weeks after a fracture [40]. The cytokines that are cornerstones in the resorption of calcified cartilage (RANKL, MCSF, and OPG) display a diminished expression in bone remodeling; in contrast, the expression of IL-1, IL6, and TNFα rise in bone marrow and is implied in this process by certain mechanisms yet to be understood [60] [44].

2.1.3. Preclinical models for bone healing

Although bone possesses an excellent spontaneous healing capacity, failures to achieve a bony union occur in the context of an insufficiency of intrinsic regenerative components, poor soft tissue coverage, unfavorable vascular supply, suboptimal surgical management, or biomechanical instability [61] [4] [62]. The resultant bone defects impose severe physical, psychosocial, and economic burdens on patients that need to be addressed by the joint efforts of clinicians and researchers. To explore novel strategies or therapeutics targeting the bone defect derived from a failed healing, researchers need to choose proper preclinical models that can simulate the pathophysiological conditions of the relevant clinical scenarios honestly.

The clinical scenarios simulated by the animal models that are commonly used in bone regeneration research include: 1) regular fracture healing, 2) segmental bone defect with subtypes of non-critical-sized defects and critical-sized defects, and 3) delayed fracture union or non-union [63]. The so called “critical-sized defect” is defined as the smallest size intraosseous wound in a particular bone and species of animal that will not heal spontaneously during the lifetime of the animal or as a defect that shows less than 10 percent bony regeneration during the

lifetime of the animal [64]. The geometrical dimension of the defect is the most emphasized aspect in this model, although many other systemic or local factors may result in an incomplete healing of bone defect, for example, bone structure, vascularization, soft tissue coverage, mechanical stability, fixation, nutrition, and age. So far, no consensus exists as to the minimal size that renders the defect "critical"; however, a defect with a length of 2–2.5 times greater than the diameter of the injured long bone or a diameter greater than 4mm in the flat bone has been accepted as "critical-sized" [65]. In contrast, the model of fracture non-union is based more on the true deficiency of osteogenic signaling, unfavorable mechanical environments, and a loss of cellular response, rather than the defect size. Besides generating a fracture or osteotomy, other procedures—including soft-tissue damage, periosteal disruption, cauterization of bone ends, perioperative irradiation, and so on—have been reported to be utilized to generate the models of fracture non-union [63, 66].

The selection of an animal model(s) is dictated by the availability of multiple objects for observation during a relatively short period, lower costs for acquisition and care, acceptability to society and ethical organizations, and so on [67 68, 69]. For the purpose of marketing newly developed therapeutics or drugs, the FDA requires a demonstration of efficacy in more than one animal species expected to react with a response predictive for humans, unless the effect is demonstrated by a single animal species that represents a sufficiently well-characterized animal model for predicting response in humans. Thus, the proper selection of a species is the primary step in providing convincing evidence for the potential of the therapeutics under investigation to benefit human beings. According to the literature, the choice of species for orthopaedic research includes rats (36%); mice (26%); rabbits (13%); dogs (9%); primates (3%); sheep, pigs, and cats (2% each) [63]. Large animal models are advantageous for several reasons: the similarity of

anatomical bone structure, analogous biological responses, comparable mechanical load, and relative ease in applying multiple postoperative management strategies [63 69]. Nevertheless, small animal models, like rodents, are extremely useful in pilot studies that aim to prove the efficacy of newly developed therapeutics or drugs at the lowest cost possible; furthermore, the vast array of genetically engineered rodents that already have been used as valuable *in vivo* tools to illustrate substantial pathophysiological mechanisms at the molecular and genetic levels [70 71] makes rodent models desirable.

2.2. Bone substitute for skeletal reconstruction

Several surgical interventions developed during the past decades, utilizing endogenous regenerative mechanisms, have successfully expedited the healing process of bone defects, especially those that are considered to be critical-sized defects and larger than 5 cm. Recently, limb lengthening with an external fixator (e.g., Illizarov fixator and monolateral external fixator), also known as internal bone transport, has been an often used surgical intervention [72]. This intervention is actually comprised of two distinct biological processes: distraction osteogenesis and transformational osteogenesis. The former occurs at the trailing end of the transport bone to bridging the osteotomy site, while the transformational osteogenesis occurs at the pathological site (e.g., non-union) under mechanical stimuli [73]. Promising for healing large scale bony defects, correcting limb deformity, and facilitating soft tissue wound care, this technique requires lengthy treatment, sufficient surgical experience, and the patient's compliance; and it is commonly complicated by fixation loosening, pin track infection, and joint contracture [74]. Intramedullary nails used alone—known as Intramedullary Skeletal Kinetic Distractors (ISKD)—or those used with the aid of an external fixator have been applied in limb lengthening procedures to reduce the fixation period of the external fixator and improve patients' comfort [75]. Nevertheless,

complications such as deep infection, premature consolidation, device failure, and non-union have been common in patients who receive this type of treatment [76].

Even though remarkable progress has been made in the development of fixative instruments and surgical techniques, with approximately 500,000 bone grafts performed in US annually, bone substitutes often are required for filling bony defects in skeletal reconstruction surgeries to recover skeletal integrity [77]. Bone substitutes need to satisfy certain criteria for clinical applications. First, neither the bone substitute nor their degradation products can be toxic; compatibility and a lack of immune reactions are among the basic requirements for any type of implant used *in vivo*. Second, bone substitutes should sustain mechanical support and display mechanical similarity to the host cortical bone in terms of toughness, modulus of elasticity, and compressive and tensile strength [78]. Third, ideally, the following biological capacities should be present: 1) osteoconduction, which guides the migration of MSC and the ingrowth of vascular tissue toward the inner structure of the bone substitutes [79], 2) osteoinduction, which induces osteogenitor cells to differentiate into osteoblast and thus stimulate bone formation [79], and 3) osteogenesis, which encourages *de novo* bone formation by the osteoblasts originating from within the bone substitutes [80]. Thus, the osteoprogenitor cells are required to attach the implant materials, survive the transplantation procedures, proliferate *in vivo*, and differentiate into osteoblasts.

Additionally, osteogenesis and angiogenesis are known to be coupled processes during physiological bone formation as indicated by the knowledge of cellular and molecular events in bone development and fracture healing [49] [22]. Thus, the capabilities of the bone substitute to induce blood vessels ingrowth and support sustained vascularization are essential for robust bone formation, which is especially indispensable regarding the healing of critical-sized bone defects

and nonunion [50]; in addition, vascularization dramatically influences the degradation or integration of engineered bone substitutes [81 82]. As mentioned previously, vascularization serves as the most efficient mechanism of mass transportation so to preserve the viability of the reparative cells that are distributed throughout the bone defect, since the simple diffusion of nutrients from the peripheries is insufficient to nurture the cells in the center of large defects. Both mass transportation and tissue growth require the structure of bone substitutes to be sufficiently porous with optimal pore size and interconnections. Previous research work has revealed the ideal pore size for the efficient mass transportation for cell survival to be at least 100μm and 200–350 μm for bone tissue in-growth [83 84].

The bone substitutes used in reconstruction surgeries can be either biodegradable or nondegradable. The nondegradable substitutes, such as metal prosthesis for orthopaedic and dental applications, can be anchored in host bone by a mechanism called *osseointegration*, which is defined as a direct structural and functional connection between living ordered bone and the surface of a load-bearing implant [79 85]. Successful osseointegration—which requires the growth of recipient bone onto, as well as into, an implant—eliminates the progressive relative movement between the non-vital implant and the bone with which it is in contact, which supports the long-term survival of the implant [86]. So far, osseointegration has provided the fixation mechanism of many non-vital and nondegradable medical prosthesis that are used in dental, orofacial, and orthopaedic surgeries.

Currently, the common bone substitutes used in reconstruction surgeries are harvested bone graft, synthetic material, or a combination of these substitutes. Advancements in material science and cell biology have made possible the manufacture of synthetic bone substitutes that can provide healing mechanisms and bear mechanical loading. Predictable synthetic bone

substitutes are needed to replace bone grafts for the increasing clinical scenarios that are being predicted for the coming decades.

2.2.1. Bone grafts

A *bone graft* is the bone tissue commonly harvested from the patient's own healthy bone (autograft), from the donors of the same species (allograft), or from a species other than human (xenograft). The cortical component of a bone graft is featured by an ideal mechanical property suitable for load bearing, while the cancellous or the cortical-cancellous component possesses one or more innate biological components: an osteoconductive extracellular matrix that induces bone ingrowth, osteoinductive molecules that direct osteoprogenitors to differentiate, and osteogenic cells that contribute to *de novo* bone formation given the proper microenvironments [9].

2.2.1.1. Autograft

Autologous bone graft is the second most common transplanted tissue in the United States and remains the gold standard for bone grafts with over half a million procedures done per year [87]. Its success in treating the orthopaedic conditions that are characterized by bone defect can be attributed to its superb biological capacities in osteoconduction, osteoinduction, and osteogenesis [88]. The common donor sites include the anterior or posterior iliac crest, proximal tibia, distal femur, fibula, and distal radius [87]. Recently, a so called Reamer Irrigator Aspitator System (RIA) has made it possible to harvest large amounts of cancellous bone from the medullary canals of long bones [89]. With respect to bone defects greater than 5mm that need to be reconstructed, the routine autograft is not advocated because it frequently leads to resorption and mechanical failure [12]. Alternatively, vascularized bone grafts (VBGs) or a bioactive pseudomembrane technique in combination with autograft (Masquelet technique) are

recommended options to regain the structural integrity of a large bone defect [12]. The commonly used VBGs are iliac flap, serratus anterior-rib flap, and fibula flap that may have the skin, facia, and muscle components attached. Since they can be anastomosed to the recipient sites, they are superior to other grafts for treating the combined defects of bone and soft tissue, which is especially useful in maxillofacial reconstructions [90]. The Masquelet technique is a two stage procedure: first, a methylmethacrylate cement spacer is inserted into the defect to form a pseudo-synovial membrane, and second, the methylmethacrylate cement spacer is removed followed by a filling with a fresh cancellous autograft [91]. The satisfactory outcomes of these two modalities are heavily dependent on the individual surgeon's experience and technique. Another special autologous bone graft is a devitalized bone graft that has been used after bone tumor reconstruction. A *devitalized autograft* is the resected bone segment in which the tumor cells have been killed by extracorporeal procedures, such as irradiation, autoclaving, pasteurization, and freezing and thawing in liquid nitrogen to name a few [92]. Unfortunately, these non-viable bone grafts often undergo resorption after re-implantation, so several researchers have explored the modality of increasing their survival after implantation by means of co-transplanting vascularized bone grafts as discussed above [93].

The application of autograft is restricted by the limited availability of resources and complications. The most common complications include infection, hematoma formation, fracture, hypertrophic scar, chronic donor site pain, and sensory disturbance [94]. The reported incidence of donor site morbidities varies from 6% for harvesting from iliac crest to 19.37% for the RIA harvesting technique [95]. The risk of complications can be reduced by following the principles of harvesting techniques and avoiding technique errors [95]. Little evidence is available for the reparative potential of the autologous bone graft in the elderly [6]. The decreased

mechanical strength of the scaffold has been noticed by surgeons; in addition, the age-related decline of bone forming cells and growth factors also has been documented in the literature [6]. As a result, aging may impair the reparative capacity of the autologous bone graft.

2.2.1.2. Allograft

In 1879, the first allograft was implanted in a 4-year-old child to replace the proximal two thirds of the humerus [96]. Today, 300,000 procedures of allograft implantation are done annually worldwide, which is 25 times the rate of kidney transplantation and 100 times the rate of heart transplantation [21]. In the US, allograft accounts for one third of all bone grafts used. Allogeneic bone grafts harvested from living donors, multi-organ donors, or cadavers can be used when they are fresh, frozen, or freeze-dried (lyophilized) [97]. Commercially available allografts are manufactured in different forms to meet a diversity of clinical demands: a massive structural allograft for mechanical support, such as osteochondral and whole bone-segment; and a cancellous-bone-like non-structural allograft to provide biological stimuli, such as morsellized cancellous chips [11]. The major advantages of the allogeneic bone graft are its relatively unlimited availability, flexibility of shape and size, and low donor-site-related morbidities [11].

When used fresh or frozen, allografts trigger a high rate of immune rejections that in turn lead to graft failure, although high osteoconductive and osteoinductive properties are well preserved [98 99]. Communicable infection, such as HIV and HBV, can potentially be transmitted through allograft transplantation even though rigid protocols are used to screen donors and test pathogens [99]; an especially higher risk is present when bone grafts are harvested from cadavers, since this harvesting may lack the appropriate monitoring. As such, various processing modalities for allografts have been established for the purposes of mitigating antigenicity and

sterilization, such as physical debridement, ultrasonic washing, treatment with ethylene oxide, antibiotic washing, and gamma irradiation for spore elimination [100]. However, the processing may lead to diminished immune reactions and infection transmission, and to impairments of biological and mechanical characteristics [100]. Even though freeze-dried allografts elicit minimal immune reactions, they are devoid of osteogenic potential. The osteoinductive and osteoconductive capacities of freeze-dried allografts also are inferior to those of fresh and frozen grafts[21].

Bone allograft can be either mineralized or demineralized. The mineral phase of an allograft allows good osteoconduction by providing a hydrophilic framework that supports creeping substitution by bone ingrowth [98 101]. The implanted mineralized bone allografts have been reported to contain osteocytic lacunae and a Haversian system that undergoes re-organization with newly formed bone tissue [102]. However, the osteoinductive potential is suppressed by the mineral content unless at least 40% of it is removed to expose the biologically active signals embedded in the bone matrix to MSC [97]. The first reported use of the decalcified bone—known as demineralized bone matrix (DBM)—used to fill osseous defects was in the 19th century [103], and it has evolved into various ready-to-use commercial products [97]. As an allograft option, DBM is produced by following several steps: procure from donors, debridement, antibiotic soak, morselization, acid demineralization, several rounds of freeze-drying, formulation into putties, paste or pre-formed strips, and finally sterilization and packaging. Classified by the FDA as reprocessed human tissue, DBM preserves most extracellular matrix proteins, both collagenous and non-collagenous, and growth factors, such as BMPs and TGF-βs, that provide the excellent osteoinductive capacity reported by numerous researchers [97 21]. Moreover, DBM can be combined with diverse carriers or an autogenous bone graft and molded

into various shapes and volumes to fill in the desired defective area [104]; and thus the application of DBM for bone augmentation has attracted a great deal of interest from the practitioners of orthopedics, dental, and craniofacial reconstructions [103]. Nevertheless, the sterilization of DBM imposes a significant technical challenge that requires eradicating pathogens and preserving the biological activity of bone morphogens [105]. The standards for the quantification of the osteoinductive capacities of different DBM preparations, and the optimal amount of DBM in a certain carrier formula to achieve a biological response remain to be clarified; in addition, the correlations between osteoinductive potentials shown experimental assays and their clinical significance need to be established as guides for the application of DBM in patients [103].

2.2.1.3. Xenograft

A xenogenous bone graft is derived from a different species other than the recipient. Several types of bone xenografts are available as ready-to-use products, and numerous research groups continue to explore their application for bone augmentation. Typical examples include the bone grafts harvested from bovine bone (Bio-Oss, Osteohealth, Sherly, NY) [106], porcine bone [107], equine bone (Osteoplant Flex, Bioteck Srl, Vicenza, Italy) [108], coralline hydroxyapatite (ProOsteon, Interpore International, Irvine, CA) [109], as well as the products derived from chitosan [110], gusuibu [111], and red algae [112]. By hydrothermal exchange, coral calcium phosphate is converted to crystalline hydroxyapatite with pore diameters between 200 and 500μm, which are comparable to trabecular bone structure [113]. Similar to allografts, xenografts are processed after harvesting—by enzymatic digestion or other methods of deproteinization—to eliminate antigenicity so to reduce immune rejections and enhance the survival of the grafts [19]. The mineral phase and architecture of xenografts are fairly well preserved during processing as are the osteoinductive and osteoconductive potentials and the mechanical properties. However, the

absorbability of xenografts has been shown to be significantly slower than autografts. In some cases, the necessary use of immunosuppression will inevitably raise concerns about its related adverse effects[114].

2.2.2. Bone tissue engineering

In the past 3 decades, extensive studies on the fabrication of engineered bone substitutes, which is known as bone tissue engineering (BTE), emerged to overcome the shortcomings, limitations, and complications of the currently utilized bone grafting. BTE is a combination of cells, biomaterial scaffolds, and suitable biochemical factors that can improve bone function or replace bone tissue[115]. The most obvious advantages of this approach are the unlimited supply of bone substitutes, avoidance of donor site morbidities, and elimination of pathogen transfer [83]. The success of BTE is rooted in the collaborative efforts of material engineers, biomedical scientists, and clinicians. The key components that synergistically enhance bone repair and regeneration include: 1) a biomaterial scaffold that mimics the extracellular bone matrix, houses osteoprogenitor cells, releases osteogenic signals, and supports endothelium invasion [116 117], 2) osteogenic and angiogenic cells that coordinate the laying down of bone matrix and the forming of the internal architecture [118], and 3) morphogenic signals that elicit the expression of the desired cellular phenotypes [119 120]. To date, the employed strategies in clinical applications include: 1) the direct injection of bolus cells into the lesion, 2) the fabrication of a certain tissue structure *in vitro* followed by transplantation, and 3) the scaffold-based delivery system of signalling molecules [82]. The bone substitutes generated by using BTE strategies can be conditioned or adjusted in a customized manner to cater to the unique needs of the bone reconstruction of each unique case. Since the prevalence of bone disease is increasing due to numerous changes in population composition and life style—such as aging, obesity, lack of

physical activity, and prolonged life expectancy resulting from better treatments of once lethal diseases—it is predictable that BTE research will expand to meet the global and escalating needs for novel bone grafts and to address the specific challenges of individual cases.

2.2.2.1. Biomaterial

Biomaterials play a pivotal and diverse role in BTE to achieve desired biological performance. First, biomaterials govern the mechanical properties of BTE constructs and primarily sustain load bearing [121] [122]. Serving as scaffolds, biomaterials establish microenvironments similar to physiological ones, which facilitate the delivery of oxygen and nutrients and the excretion of waste products [84], as well as supporting various cellular activities including the attachment, migration, proliferation, differentiation, and deposition of the secreted extracellular matrix [84]. The biomaterial scaffolds also are suitable vehicles to immobilize various growth factors and to deliver biological therapeutics *in vivo* [120]. In conclusion, biomaterials are not only a substitute for an injured tissue to perform an instant biological or mechanical function but also serve as guidance for tissue repair in long-term.

The criteria for the ideal biomaterials used in BTE have evolved over the past 5 decades, and the commonly used materials currently used today are polymer, ceramics, metal, or a combination of these materials [78]. Following is a list of several essential considerations regarding biomaterial design, selection, fabrication, and utilization for BTE:

1) *Mechanics*. At the early stage of utilizing synthetic materials in bone reconstruction, the candidacy of the material is primarily dictated by its mechanical properties [123]. The optimal mechanical profile should match those of the replaced bone: insufficient rigidity

may cause mechanical failure and implant fracture; on the contrary, too much rigidity could lead to bone loss due to stress shielding [124].

2) *Chemistry*. The biomaterials used in BTE and their degradation products should be neither toxic nor allergenic and produce only minimal adverse effects [125]. The metals used in BTE have a robust resistance to corrosion and release a minimal amount of ions [126].

3) *Biology*. When biomaterials were first used, they were merely required to be bio-inert after implantation—having very few interactions with physiological microenvironments and releasing only a minimal amount of ions or other particles [125]. Later, biocompatibility and bioactivity became the new standards to optimize the bone tissue formation that is induced directly at the interface between the bone and the implant in contrast to the nonspecific fibrotic response and foreign body reaction that often occurs around implanted synthetic materials [123]. Some researchers have suggested that bioactivity is related to the formation of a carbonated hydroxyapatite (CHA) bone-like layer on the surface of an implant that is capable of adsorbing growth factors and enhancing osteoblast differentiation [127]. Thus, the mineralization of biomaterials and the modifications of the surface by adsorbing proteins or tethering polymers and macromolecules are the available treatments to enhance the biocompatibility and bioactivity of materials used in BTE [123]. The resultant binding between bone tissue and implant is known as the aforementioned *osseointegration*, a process that also can be governed by the modifications of the surface features of biomaterials, including wettability, roughness, and electrostatic charge [128]. Recently, advancements in the field of material science and technology have made it possible for biomaterials to induce specific

tissue and cellular responses through their interactions in the interface at the molecular level [129]. With respect to the use of biodegradable materials, the rate of bone regeneration should be comparable to that of scaffold degradation and remodeling so to maintain the mechanical strength of these biodegradable materials and avoid mechanical failures [129].

4) *Porosity*. The material scaffolds featuring highly interconnected porous networks with a size of 100–350μm have been reported to be ideal for bone tissue formation and vascularization, since they satisfy the requirements for efficient mass transportation and osteogenic and angiogenic cells migration [130]. Porosity also can influence the bioperformance of biomaterials by effecting degradability and mechanical properties [131].

2.2.2.1.1. Polymer

According to their origins, polymeric materials used for bone reconstruction can be mainly divided into two classes: 1) *Natural polymers*. Among the most commonly used naturally occurring polymers are proteins and polysaccharides, for example, collagen, chitosan, hyaluronic acid, alginic acid, and fibrin [132]. Chemical modifications made to natural polymers can give birth to new polymers such as cellulose acetate [132]. 2) *Synthetic polymers*. This category encompasses a huge range of materials utilized as bone tissue analogues, for example, polymethylmethacrylate (PMMA), polyglycolide (PGA), polylactide (PLA), and poly (3-caprolactone) (PCL) to name only a few [123].

Control of the mechanical properties of synthetic polymers through adjustments in their chemical composition and manufacturing conditions enables their use as fixation materials [133]. Self-reinforced PGA, self-reinforced PLA, and other synthetic polymers have been fabricated into fixation apparatus such as staples, pins, or screws to fix fractures, arthrodesis, or osteotomies

without the necessity for second surgery for fixation device removal [134]. The degradability of PLA and its mechanical rigidity similar to bone has led to its application as an interbody cage for spine fusion [135]. PMMA was introduced in the late 1950s by Charnley as material for anchoring hip prosthesis, which has achieved a satisfactory outcome in the long term [136]. More recently, PMMA was injected in vertebroplasty and kyphoplasty to augment the strength of weakened vertebral bodies; in addition, it often has been used as a temporary "space filler" in staged surgeries to maintain anatomical morphology [137]. Other bio-inert polymers with proper mechanical properties have been successfully used to replace diseased parts of the skeletal system. For example, UHMWPE and silicon rubber have been made into the liners for total joint replacements—the former had been used for weight bearing joints, such as knee and hip joints [138], and the latter for small joints, such as phalangeal joints [139].

The polymeric materials that are both biodegradable and bioactive are advantageous candidates for designing BTE composites. Many natural polymers show innate bioactivities due to their functional groups and binding sites on the surface that favor the attachment and spreading of molecules and cells [140]. However, concerns related to natural polymers include immunogenicity, risk of disease transmission, poor handling, and weak mechanical properties [141]. Lacking the disadvantages innate to natural polymers, synthetic polymers are generally void of bioactivity per se [141]. Several strategies have been proposed to enhance the bioactivities of polymeric materials for the purpose of generating competent bone analogues. Biomolecules that mimic the functional domains of the extracellular matrix protein or growth factors can be linked to the polymeric materials by physical adsorption or chemical bond [142], and therefore increase the specific tissue response around the implant. Alternatively, polymeric implants can be reinforced with ceramic phases directly *in vitro* or can be chemically treated to induce mineral

deposition *in vivo* to boost their bioactive potentials to expedite peri-implant bone formation [143]. Hydrogels— such as chitosan, PHEMA, and hyaluronic acid—possess a three-dimensional structure that is maintained by hydrogen and ionic bonds and a large volume of water within the internal structure [144], which creates a potential for carrying reparative cells and signalling molecules in the polymeric construct [145, 146]. In addition to being used in the repair of cartilage and intervertebral discs, hydrogels also provide an avenue for augmenting bone mass through minimally invasive injections [147]. The biodegradable polymers are degraded by hydrolysis and enzymatic attack with the final by-products being eradicated by metabolism reactions [132]. Examples of biodegradable polymers include PGA, PLA, PDS, PCL, PHB, polyorthoester, PHEMA, hyaluronic acid, chitosan, and other hydrogels. The ideal rate of implant degradation that should match the rate of new bone formation can be changed by adjusting the multiple features of the polymer material, such as crystallinity, molecular weight, thermal transitions, and porosity to name only a few [123].

To date, numerous sophisticated technologies have been developed to engineer bioactive and biodegradable polymeric scaffolds with predestined geometrical properties, porous internal architecture, and preferred surface characteristics. Some of the commonly used techniques are phase separation, gel casting, solvent casting and particulate leaching, gas saturation, and most recently, 3D printing [132]. Solid Free Fabrication (SFF) is the core technology of 3D printing that enables a layer-by-layer manufacturing process and the control of the three dimensional architecture by Computer-aided Design (CAD) data [148]. Each of these processing techniques confers the scaffold with a distinct combination of properties, so the selection of these techniques is dictated by the final application [132].

2.2.2.1.2. Ceramics

Since the 1970s when ceramic materials were first being investigated for use as a bone substitute [125], many formulas have been developed for using synthetic bioceramics as substitutes for bone grafts, including the commonly used alumina, zirconia, bioactive glasses (BGs), calcium phosphate preparations (synthetic HAs, α- and β- TCPs, biphasic calcium phosphate [BCP]), calcium sulfate, and glass-ceramic compositions [123]. Control of the conditions of the manufacturing process has enabled the generation of a multitude of ceramic materials characterized by distinct physical, chemical, and biological properties that dictate their potentials for clinical applications [123, 140 149]. Alumina and zirconia, for example, are characterized by high rigidity and corrosion resistance as well as low friction and wear coefficients, and thus became the pioneering ceramics used for the articulation components—femoral head and acetabulum—for total hip arthroplasties [150]. However, their low fracture toughness and high elastic modulus have raised concerns about mechanical failure and serious stress shielding in the long-term [151].

Unlike alumina and zirconia's resistance to ion exchange and their lack of bone apposition, the vast majority of bioceramics being studied as bone substitutes—belonging to either BGs or CaP—have displayed excellent capacities for osteoconduction and osseointegration [77], so they are widely used as defect filler to induce bone regeneration from surrounding tissues [149] and as complementary components for other biomaterials made from polymers or metals to enhance their bioactivity [143]. The structural and surface features of these bioceramics can be similar to the mineral phase of bone, which contributes to the direct bony bridging of the implant to host bone in the absence of fibrous tissue at the interface [152]. Several BGs primarily formed by the silicon dioxide (SiO_2) network and complemented by some modifying oxides and glass-ceramic compositions have shown more competent osteoconductive

capacity than HA [153]. Si and Si-OH groups on the surface provide nucleation sites for the crystal cells of CHA, a bone-like layer formed on the surface of bioactive material, and thus facilitate the growth of apatite layers [127 154]. In addition, a negative zeta potential brought about by abundant OH^- groups lends extra help to nucleate CHA and precipitate Ca^{2+} [154]. While the bone formation proceeds, the ceramic implants undergo degradation or remodeling at a rate that is related to the chemical formulation and preparation method. HA ($Ca_{10}(PO_4)_6(OH)_2$) is subjected to a slow degradation with the majority of it directly incorporated into regenerated bone tissue while β-TCP ($Ca_3(PO_4)_2$) completes the degradation [123]. Similarly, the degradability of BGs can be modulated by altering the portion of sodium oxide in the formulation according to specific needs [155].

From a mechanical point of view, the commonly used bioceramics, including HA, TCP, and BGs are weak in tensile and compressive strength and have a low fracture toughness that can be worsened by the degradation process [156]. Thus, the application of ceramic material in bone reconstruction necessitates either rigid fixation or the lesions being non- or low weight bearing [123 70]. So far several ceramic materials approved by the FDA have been used as bond void fillers in periodontal disease [157] and orthopaedic pathologies [158]. Another promising approach for treating skeletal defects is bioceramics used as cement [159]. Glass ionomers and a range of CaP preparations (amorphous CaP (ACP), α-TCP) can be delivered by injection and hardened inside the tissue with significant lower heat injuries to the host bone tissues than those incurred by PMMA [159].

Ceramic materials are being studied intensively for the fabrication of porous implants with a combination of high degrees of macroporosity (250–350 μm) and microporosity (3–8 μm) [140 160]. 3D printing technology is proving to be a powerful tool for tuning porosity and

manufacturing a ceramic scaffold with a hierarchically organized structure [161]. The exploration of dopant addition in scaffold is providing more solutions for controlling the dissolution rate, mechanical strength, and bioactivity [140 162 163]. The use of ceramic material as a supplement to a scaffold made of metal or polymer has been proved to be an efficient approach to enhance the bioactivity of an implant, promote the therapeutic potentials of biological cues, and avoid the problems caused by the shortcomings of using ceramic materials alone [140 164].

2.2.2.1.3. Metal

Metallic materials have been used as either instruments for fixation and deformities correction, or as bone substitutes, such as prosthesis. In the late 1950s, Charnley utilized stainless steel for the first successful substitutive joint prosthesis for a total hip replacement [136]. Rich in Cr content, stainless steel is able to form an adherent and self-healing oxidation layer of Cr_2O_3, which is corrosion-resistant and biosafe in the *in vivo* application [123]. In addition, stainless steel is widely used for temporary fixation apparatus, such as fracture plates, screws, and intramedullary nails [165]. More recently, Co-Cr-Mo alloy has gained popularity as the candidate metallic material for joint and disc prostheses because of its excellent resistance to chemical corrosion and physical wear [166]. However, these two types of metal are problematic in terms of their much higher elastic modulus (approx. 200 GPa) compared to that of cortical bone (20–30 GPa) and their tendency to produce stress shielding in the adjacent bone [167].

Originally used in aeronautics, titanium alloy is currently the most popular type of metal for implanted medical devices due to its moderate elastic modulus (approx. 110 GPa), a good corrosion resistance, and low density (approx. 4700 kg/m^3) [167]. Similar to Co-Cr-Mo alloy, Ti alloy is oxidized rapidly on its surface, and the resultant of TiO_2 layer protects it from corrosion

[168]. Furthermore, it was in the study of the Ti implant undertaken by Branemark in the 1960s that osseointegration was initially conceptualized [169]. Given these physical, chemical, and biological advantages, Ti and its alloys with different supplements are commercially available, among which CP Ti with a single-phase alpha microstructure has been made into dental implants [170], and Ti6Al4V with a biphasic alpha-beta microstructure has been used in orthopaedic applications [171]. Interestingly, NiTi alloys have been applied in staples, internal fixation devices, spine correctors, vertebral spacers, and distraction devices because they are distinct from traditional alloys in their shape memory effect [172], their ability to recover their shape when heated after being plastically deformed, and the difference in their elastic modulus between their martensitic state induced by stress (as low as 30 GPa) and their austenitic state (70–100 GPa) [172] [123]. Recently, Ti and its alloy have been designed and manufactured into various porous structures, fiber mesh, or metallic foam [173], which— according to extensive *in vitro* and *in vivo* studies conducted by numerous research groups—have exhibited excellent osteoconduction and osteoinduction and a potential for drug delivery [174] [175] [167]. Other metallic materials investigated as alternatives to titanium and its alloys include tantalum [176], aluminum, niobium, nickel, zirconium, and hafnium (10.4028/www.scientific.net/MSF.512.243).

While bio-degradable metallic bone substitutes, such as the bio-resorbable foams of magnesium, are under investigation [177], the vast majority of bone substitutes made of metal are non-degradable and require robust anchorage mechanisms to guarantee good long-term outcomes [86]. The anchorage of metallic bone substitutes can be generally categorized as either cemented or cementless as is discussed in the next section.

2.2.2.1.3.1. Cemented fixation

The first metal prosthesis for hip replacement introduced by Charnley in the 1950s was anchored by self-polymerizing polymethylmethacrylate (PMMA), also known as bone cement [136]. Over the past few decades, the structure and performance of PMMA bone cement has improved significantly thanks to the joint efforts of materials scientists and surgeons, which led to the advent of superior surgical techniques, monomer cooling, vacuum mixing, and injecting devices [178]. Despite its excellent provision of primary fixation, PMMA bone cement is not compatible with the biological fixation that occurs at a later stage [179]. Also, the difference of stiffness between a metal implant and bone imposes overstress or overstrain on the bone cement sandwiched in between, and predisposes the production of bone cement particles, which will later induce inflammation and destroy the bone structure [179]. The addition of a radiopacifier makes the PMMA cement more fragile and susceptible to mechanical failure [180]. A gap can occur because of the shrinkage of PMMA during polymerization, which can result in the subsequent loss of contact between the PMMA cement and bone or the metal prosthesis [181] [182]. Fat embolism and thermal necrosis of the bone structure also have been reported to occur while applying the bone cement, which can be due to the residual monomer entering circulation and the large exotherm produced during the polymerization of PMMA, respectively [183, 184].

2.2.2.1.3.2. Cementless fixation (osseointegration)

The anchorage of the metal prosthesis without the application of PMMA depends on osseointegration, which also is referred to as biological fixation by some researchers. This fixation approach has been shown to have an outcome in the long-term comparable to that of PMMA, but it is void of the complications and risks related to PMMA use [185]. Intensive research

into the biological process of osseointegration has provided insight into the methodologies to promote the direct binding between bone and the metal implant.

2.2.2.1.3.2.1. Process of osseointegration

The presence of a metal prosthesis and the incurred skeletal trauma from implantation initiate a biological cascade of peri-implant bone healing, which has been divided into three overlapping stages by some researchers [186]. The initial interactions between host tissue and the implant occur during the coagulation and inflammation reactions at the first stage [187] [186]. The exposure of the implant surface to blood triggers hematoma formation and, meanwhile, platelets undergo a series of morphological and biochemical changes in response to the material surface [188]. Blood clots, composed of fibrin and formed on the surface of the implant, serve as a scaffold to facilitate osteoprogenitor cells to attach and migrate onto the implant under the induction of chemotactic and growth factors secreted by the platelets and blood-borne inflammatory cells that are stabilized within the interlocking fibrin fibers [188]. Therefore, osteoconduction is the predominant event occurring at this stage. To further regulate cell adhesion and mineral binding, the MSC and osteoblasts that are able to attain the implant surface begin to deposit bone-related non-collagenous matrix proteins and create poorly mineralized osteoid tissue on the implant surface from day one after implantation [189].

The second phase of osseointegration is *de novo* bone formation [186]. As some bone forming progenitor cells complete the differentiation and stop migrating before reaching the implant surface, bone spicules advancing toward the implant surface are formed in the host bone cavity. This type of peri-implant bone formation is defined as *distance osteogenesis* [190]. In contrast *contact osteogenesis* refers to the bone development that starts from the implant surface

toward healing the host bone, mediated by the osteogenic cells that migrate onto the material surface before the terminal differentiation and deposition of osteoid tissue [190]. At 10 to 14 days after implantation, the newly formed woven bone structure by these two mechanisms restores anatomical integrity and realizes the biological fixation of the implant with a lower mechanical competence [189]. The cavities surrounded by the newly formed bone tissue are contiguous with the marrow space of the host bone and contain large amounts of blood vessels, MSC, and mononuclear precursors of osteoclasts, which play essential roles in the faster remodeling of the peri-implant woven bone into the lamellar bone [189].

The final stage is the bone remodeling stage in which the bone in contact with the implant surface undergoes adaptive morphological changes in response to stress and mechanical loading [186]. At 3 months post-implantation, the peri-implant bone is substituted by osteons that surround the implant with the highest degree of organization and mechanical competence [186]. The mature bone structure on surface of the osseointegrated implant sustains active turn-over indicated by the constant osteoid tissue formation and the presence of medullary cavities containing osteoblasts, MSC, osteoclasts, and blood vessels [189].

2.2.2.1.3.2.2. Factors affecting osseointegration

The interactions between the implant surface and biological entities, including cells and molecules, dictate the success of osseointegration [187], which is determined by the factors related to the surface properties of the implant, host bone conditions, and surgical techniques [187]. Thus, the impairment of osseointegration is attributed to the changes of these factors. Primary mechanical stability is required to be achieved during surgery or potential excessive implant mobility and micromotion can inhibit osseointegration [191]. The weakened osteogenic or

angiogenic capacities seen in many clinical scenarios—such as aging, osteoporosis, chemotherapy, radiotherapy, nutritional deficiency, renal insufficiency, and so on—lead to insufficient bone formation around the implant because of decreased cellular proliferation and differentiation [189]. The use of cigarettes and certain pharmacological agents including warfarin, low molecular weight heparins, and non-steroid anti-inflammatory drugs (NSAIDs) also exert negative effects on osseointegration [189]. On the other hand, strategies to improve and expedite the osseointegration process are under investigation, which mainly has focused on the material engineering used to improve the bioactivities of the implants [192]. The protein adsorption and cell attachment onto the implant surface, critical for the initiation of osseointegration, is affected by the chemical composition and physical properties of the implant surface, which can be modified by various physical, chemical, and biological methods [193] [194].

The commonly used materials for osseointegration include titanium and, to a considerably lesser extent, zirconia [176]. The structure of TiO_2 formed on the surface is believed to be related to the nucleation and growth of the calcium phosphate (CaP) layer, although the mechanisms are yet to be understood [195]. Coating the metallic materials with crystals or bioactive molecules is a viable strategy to improve the osseointegration of a metal implant[123]. Bioactive ceramics, including hydroxyapatite (HA) and bioglass (BG), are the common bioactive materials that can be coated onto metal implants by either physical or chemical methods [196]. The physical methods that are exemplified by the most commonly used plasma spray deposition and other techniques create a mechanical interaction between the ceramics and substrate [196]. The major drawback of this approach is the possible delamination of the coating from the implant surface[187]. Other concerns include difficulties in controlling the composition, crystallinity, and structure of the ceramic coating, which lead to the discrepancy in dissolution between the

amorphous and crystalline phases [187]. All these factors contribute to the particle release and structural failure of ceramic coatings. The philosophy underlying the chemical coating method is to obtain *in vivo* ceramic deposit after the chemical modification of the implant surface by establishing a chemical link between the metal substrate and HA- or other CaP-based coating materials [123]. Several techniques belonging to this approach are available, such as the attachment of self-assembled monolayers (SAMs), dipping in sol-gel solution, thermochemical treatment, and so on [123].

As cell adhesion and migration are mediated by the recognition of the extracellular matrix protein or specific peptide sequence—particularly the Arginine-glycine-aspartic acid (RGD) sequence by transmembrane cell receptors—it is thus proposed to functionalize the implant surface and improve bioactivity by tethering proteins or peptides to mimic the extracellular matrix (ECM) chemistry [197]. Other biomolecules, such as growth factors and cytokines, and more complex biopolymers, such as elastin-like biopolymers, can be added to implants by chemical bonding and physical adsorption [198]. With respect to this approach, the available technologies include dip-coating techniques, the formation of SAMs, and the more sophisticated “bottom-up” and “top-down” techniques to name only a few [123]. The covalent bonds between biomolecules and the salinized titanium surface can be established by using a glutaraldehyde chemistry that is based on an amino- and carboxyl-directed interaction [199] or photochemistry that requires grafting photoactive groups to the biomolecules [200].

In addition to the organic and inorganic composition of the implant surface, its physical features are critical determinants for bioactivity and bone apposition, such as roughness, wettability, and electrostatic charge [201]. The modification of the topography of the implant surface can alter these physical properties and be used to enhance osseointegration. Based on the

dimensions of roughness, the topography can be classified as *macro-scaled* ranging from millimeters to tens of micrometers, *micro-scaled* ranging from 1 to 10 micrometers, and *nano-scaled* ranging from 1 to 100 nanometers [202]. Macro-topography is primarily dictated by the geometrical design and is related to the porosity and pore size of the implant surface, which are vital for modulating the growth of vessels and bone tissue [203]. Researchers have reported a pore size above 80μm, which is associated with improved bone ingrowth in hydroxyapatite and tricalcium phosphate materials [204]. Nevertheless, both macro-topography and micro-topography determine the degree of mechanical interlocking between the host bone and the implant surface, as well as the long-term mechanical stability of the implant [205]. The surface roughness in a scale of micrometers can be engineered by a variety of methods such as plasma spraying, blasting with ceramic particles, and acid etching [206]. Several studies have found that the regulation of osteoblast differentiation and proliferation, as well as the enhancement of the osseointegration mediated by the surface roughness in a scale of micrometers, are related to the activation of the integrin receptors by the substrate [207]. Therefore, a rough implant surface favors both distant and contact osteogenesis, although distant osteogenesis predominates around the smooth implant surface [189]. Nano-scaled topography has become a source of interest for material scientists because it increases surface energy and wettability [208]. Thus, the expedited adsorption and spreading of proteins and the promotion of cell attachment facilitate wound healing and bone growth [208]. Some researchers have reported that a nano-scaled pattern can modulate cell proliferation and differentiation [209]. A fractal or fractal-like architecture exists in many biological structures and influences several biological tissue responses, so the evaluation and application of fractal architecture at the micro- or nano- scales are under investigation [202].

Several groups are exploring other strategies involving the concurrent use of biological therapeutics with metal implants. This approach is especially promising for those patients whose inherent bone healing capacities are impaired and lack bone forming cells and signals [189]. It also has been reported that additional applications of demineralized bone matrix (DBM) and bone marrow aspirate withdrawn from iliac promote the osseointegration of porous implants [210] [189].

2.2.2.2. Cells

The rationale for incorporating cells into BTE constructs is rooted in their functions: 1) secretion of osteogenic or angiogenic factors, 2) generation of temporary templates to recruit osteogenic and angiogenic cells, and 3) production of bone matrix and formation of vascular structure [115]. To promote bone regeneration, the cell-based BTE strategies take advantage of both endogenous cells that are recruited and exogenous cells that are introduced into the bony defect sites. Cellular scaffolds with or without modifications by bioactive molecules are being studied extensively in recruiting cells from host tissues, with a few of them being commercialized for orthopedic and dentistry use [83]. The simplest approach to capitalize on exogenous cells is to administrate the cell suspension directly into the lesion that undergoes self-assembly *in vivo* [211]. Although several researchers have reported promising results, this approach has not been widely used in clinical practice yet, and the ability of these cells to form high ordered bone architecture still remains a concern. Pre-seeding exogenous cells onto scaffolds seems the most promising strategy to capitalize on cells in BTE; nevertheless, it is technically complicated to obtain a uniform cellular distribution in the 3D construct, which often necessitates the application of bioreactors such as perfused cartridges, rotating vessels, and spinner flasks [212].

Before exogenous cells can be successfully used, several issues need to be addressed. The primary tasks left to finish include the recognition of cell resources, the isolation of the cells with proper identities, and the efficient expansion of these cells. Inducing and stabilizing the required phenotype of seeded cells involves adjustments of culture conditions, such as the pH value, oxygen tension, growth factors, steroids, and hydrodynamic or mechanical environments [115] Before recommending this approach for clinical use, long-term safety has yet to be thoroughly evaluated to exclude any possible immune rejections, graft-versus-host responses, and tumorigenesis to name only a few [115].

2.2.2.2.1. Stem cells

Stem cells are capable of replicating and differentiating with hierarchical potencies [213]. The totipotency features of the zygote cell, derived from the fertilization of the oocyte, enables this cell to differentiate into any cell type of the human body [214]. Pluripotency means indefinite mitosis and an ability to differentiate into derivatives of three germ layers, namely ectoderm, endoderm, and mesoderm [215]. Embryonic stem cells (ESC) are pluripotent cells located within the inner cell mass of the blastocyst. Multipotent progenitor cells, such as adult stem cells, have a gene activation potential to differentiate into multiple, but limited, cell types in response to stimuli [216]. Adult stem cells can be found in various tissues and so are more applicable cell resources for transplantation purposes. Hematopoietic stem cells donated by healthy individuals are widely used for treating numerous hematological malignancy and non-malignancy conditions [217]. Mesenchymal stem cells can be found in various tissues originating from mesoderm and serve as reparative cells to maintain tissue homeostasis [216]. Currently, mesenchymal stem cells (MSC) have drawn the attention of researchers and clinicians focused on BTE who have

conducted extensive studies using experimental models [216] and several small-scaled clinical trials for myocardium infarction [218] and fracture non-union [219].

2.2.2.2.1.1. Adult stem cells (Mesenchymal stem cells)

Adult stem cells are distributed in various types of tissues to constitute a reservoir of reparative cells for the tissues in which they reside, for example, bone marrow, periosteum, trabecular bone, teeth, dental pulp/dentoaveolar tissue, adipose tissue, skeletal muscle, tendon, amniotic fluid, umbilical cord/peripheral blood, placenta, liver, and spleen [220]. In the 1880s, Cohnheim, a German pathologist, was the first researcher to reveal the existence of non-hematopoietic stem cells in bone marrow, which later were referred to as mesenchymal stem cells or mesenchymal stromal cells (MSC) [221]. MSC originate from the mesoderm sclerotome condensations at the embryo stage and are distributed in various tissues as mentioned previously [222]. Although no consensus exists as to the identity of MSC, the criteria stipulated by the International Society for Cellular Therapy is accepted for identifying human MSC for the purposes of this present thesis: 1) adherence to plastic, 2) formation of fibroblast like colonies, 3) tri-lineage differentiation *in vitro* including chondrocytes, osteoblasts, and adipocytes, 4) positive expression of CD73, CD90, and CD105, and 5) negative expression of CD45, CD34, CD14, CD11b, CD79a, CD19, and HLA-DR surface molecules [223].

The idea of applying MSC in BTE is favored by their numerous biological properties. First, MSC are able to mobilize and migrate toward the injured loci under the guidance of the released chemokines and cytokines, which interact with the surface receptors expressed by MSC—such as CCR1, CCR7, CCR9, and CXCR4-6—and as a consequence, stimulate β-actin filament reorganization and cell motility [224].

The potential of MSC to differentiate into multi-lineage mature cells that are able to produce an extracellular matrix is the paramount reason why they can assume the responsibility of tissue repair. The advancements made in developmental biology during the past decades have led to the recognition of more transcription and growth factors that direct MSC differentiation in these lineages. With respect to myogenic differentiation, the important molecular signals include the basic helix-loop-helix MYOD, myogenin, MYF5, and MRF4 [225]. The fatty acid-sensing peroxisome proliferator-activated receptor-γ (PPARγ) drives adipocyte differentiation [226]. Sox9 has been recognized as the most important regulator for chondrogenic differentiation [227], while osteoblast differentiation requires BMPs, WNTs, Hedgehog, RUNX2/Cbfa1, Osterix, and MSX2 [228]. The application of these key molecules as supplements to the culture medium or genetic engineering can control the direction and rate of MSC differentiation. Interestingly, the stromal vascular fraction of adipose-derived stem cells (SVF of ADSC) and bone marrow-derived MSC (BM-MSC) have been recognized as progenitor cells for angiogenesis, and can be induced to differentiate into endothelial cells *in vitro* and have been found to enhance vascularization after transplantation *in vivo* [229] [230]. In addition, MSC can promote vascularization by secreting VEGF, bFGF, and other angiogenic factors to exert paracrine actions on surrounding cells [231]. Thus, the capacity of MSC to enhance both osteogenesis and angiogenesis makes them promising candidates for bone engineering.

Although the frequency of MSC is usually low—for example, 1/10,000 to 1/100,000 of bone marrow mononuclear cells [232]—their outstanding proliferation capacity can overcome this shortcoming and guarantee the applicability of several times of passage and sufficient numbers of cells for clinical use. Also, the breakthroughs made in cell culture technology have expedited the process of translating *in vitro* expanded cells to clinical applications. 3D culture in

bioreactors has been showing its superiority to traditional 2D culture in terms of more efficient mass transportation, a higher yield of cells, and the required cellular phenotypes induced by mechanical stimuli [233]. The advent of the use of autogenous serum and allogeneic serum, as well as serum-free medium supplemented with growth factors, has addressed concerns about immune reactions and the transmission of infectious pathogens related to the use of bovine serum [234].

Some researchers have suggested that allogeneic MSC can be used for transplantation without the ablative conditioning that is routinely done for hematopoietic stem cell transplantation [235 236]. Indeed, several researchers have reported their successful experiences with using xenogenous MSC for tissue repair in experimental models [237 238]. The immune privilege embodied in the MSC is attributed to secreted immunomodulatory factors such as IL-10—as well as the absence of antigenic molecules, such as the HLA class II histocompatibility antigen—expressed on their surface [239 240]. Furthermore, the majority of osteoblasts derived from the transplanted MSC only survive for a short period of time *in vivo* before being surrounded by minerals and undergoing apoptosis with a minority being embedded in the newly formed bone structure as osteocytes that cannot impose a persistent immunological threat [241]. Some researchers have implied that the production of bone by the transplanted MSC is not as important as their secreted growth factors or cytokines that improve microenvironments and exert trophic effects on endogenous cells [235].

The strong viability exhibited by MSC allows extensive *in vitro* manipulations before their *in vivo* applications. In addition to being used as unfractionated fresh bone marrow cells, MSC are often subjected to concentration, expansion by cell culture, induction of differentiation, and genetic modifications using transfection or transduction. Recently, some researchers have proposed mixing MSC with platelet rich plasma (PRP) before their administration [242]. Also,

MSC are compatible with various types of delivery approaches, ranging from direct injection to implantation with biomaterial vehicles [219].

2.2.2.2.1.2. Embryonic stem cells (ESC)

Embryonic stem cells (ESC), by definition, are harvested from embryos that are formed following fertilization until the ninth week of gestation; however, the *in vitro* fertilization technique enables the development of extra embryos to serve as resources for ESC to mitigate the ethical debate regarding their usage [243]. Being pluripotent, ESC can differentiate into all the lineages present in mature bone—including osteoblasts, osteoclasts, vascular cells, and nerve cells—for bone regeneration [215]. However, ESC are so plastic that their *in vitro* differentiation is inconsistent and disorganized [244]. To expand ESC, complex proliferation culture systems are required, which include a specific medium with the addition of various growth factors, feeder cell layers, and/or coated culture plates [245]. Some researchers have found that the 3D scaffold is superior to the 2D culture plate for directing the osteoblastic differentiation of ESC for BTE [246] [244]. In addition, proliferating ESC usually possess abnormal karyotypes, which are believed to be one of the mechanisms that underlie the teratomas formation [247] [248]. Therefore, the safety issues involved with the *in vivo* use of ESC, such as tumorigenicity and immune incompatibility, have yet to be assessed. Last but not least, the inevitable destruction of fertilized human embryos during the harvesting of ESC will continue to be an ethical issue that impedes the research and clinical use of ESC [249].

2.2.2.2.1.3. Induced pluripotent cell (iPC)

In 2012, John Gurden and Shinya Yamanaka won the Nobel Prize in physiology and medicine for their contribution to research involving the induction of adult somatic cells into

pluripotent cells [250]. Mouse fibroblasts can be converted to pluripotent cells with morphological, immunological, and biochemical properties similar to those of ESC by retrovirally introducing gene products of Oct3/4 (also called Pou5f1), Sox2, c-Myc, and Klf4 [251]. This discovery provides a promising solution to circumvent the ethical controversies related to ESC usage. The pluripotency of iPC potentiates their applications in the tissue engineering of various organs and tissues that originate from all three embryonic germ layers [252]; however, the epigenetic memory of the original differentiated somatic cells may influence the differentiation potentials of iPC and the functionalities of the tissue they form [253]. Currently, great efforts are being made to characterize iPC, and further studies on their applications in preclinical models are warranted.

2.2.2.2.2. Osteoblast

Osteoblasts can be harvested primarily by mechanical mincing and the proteinase digestion of bone samples [254 255]; alternatively, they can be obtained by an *in vitro* induction of MSC into preosteoblasts or osteoblasts [256]. Although osteoblasts exhibit inferior potentials for proliferation and mineralization *in vitro* when compared to MSC, their osteogenic capacity is superior to those of MSC after being transplanted *in vivo* [257]. Osteoblasts can be delivered in biomaterial vehicles, and various aspects of their activity—such as adhesion, spreading, migration, proliferation, and matrix synthesis—can be regulated by modifying the biomaterial scaffold, for example, by tethering adhesion ligands and inoculating growth factors [258]. The main issue for osteoblast use in BTE is their short life span, which necessitates continuous replacements to maintain the cell population [259 259].

2.2.2.2.3. Endothelial cells (EC)

It is a well-established doctrine that both angiogenesis and osteogenesis are closely coupled during bone repair, and are equally important for BTE. Efficient mass transportation as well as cellular viability in any tissue with a thickness over 100–200μm is dependent on sufficient vascularization [260]. Researchers believe that neovascularization is a process of tubular structure formation through the proliferation of endothelial cells from existing blood vessels [261]. In addition, several *in vitro* studies that co-cultured MSC and EC have indicated that EC increase the osteogenic markers expressed in the MSC [262]; while MSC promote tubular structure formation by EC by secreted factors such as VEGF and direct interactions with cell membrane proteins and gap junctions [118 263]. Thus, endothelial cells that can be harvested from the umbilical cord vein (HUVEC) and dermis by collagenase digestion have been explored for their potential for enhancing the osteogenesis of BTE. Researchers have reported that pre-seeding EC on a scaffold is an efficient way to engineer primitive 3D vascular networks *in vitro* and that the co-transplantation of EC and MSC has resulted in significantly more bone formation *in vivo* than the transplantation of either of them alone [264]. However, mature EC have demonstrated significant apoptosis after transplantation, which would impair their clinical applicability [265].

The existence of endothelial progenitor cells (EPC) was first described by Isner and Asahara in 1997 [266]. They are found in bone marrow (BM-EPC), peripheral blood (PB-EPC), umbilical cord blood, and the spleen to name only a few locations [267], with the bone marrow and peripheral blood being the most commonly used origins. The isolation of EPC can be achieved by using an adherence culture and mononuclear sorting that recognize the putative cell markers of EPC including CD34 and CD31 [268]. EPC can be induced to proliferate, migrate, or differentiate into cells that line the lumen of blood vessels by supplementing the culture systems

with EFG, bFGF, and VEGF [269]. Similar to mature EC, EPC enhance the osteogenic potentials of an engineered bone construct by not only improving perfusion but also the crosstalk to MSC to enhance osteoblastic differentiation by means of paracrine and direct contact [270]. Recently, some researchers have proposed that EPC per se are osteogenic, and they are able to de-differentiate into the common ancestors of EPC and MSC and re-differentiate into MSC and final osteoblasts [271]. Comparisons between BM-EPC and PB-EPC have pointed out the former are at an earlier stage and express the early hematopoietic marker CD133, whereas the latter display typical endothelial markers [272 273]. PB-EPC possess superior angiogenic potential in a 3D ECM mimicking scaffold; in addition, PB-EPC have a higher expression of osteogenic and angiogenic markers, including BMP2, VEGF, and ALP than BM-EPC when co-cultured with MSC [274]. Considering the donor site morbidities related to BM-EPC harvesting, PB-EPC appear to be a more promising cellular candidate for the vascularization strategy of BTE.

2.2.2.2.4. Bone marrow aspiration (BMA)

To circumvent the complexity of isolating and the *in vitro* expansion of MSC with their related higher expenses, fresh bone marrow aspiration (BMA)—with or without concentration—has been explored as an alternative to using MSC for BTE [211]. BMA is a viable source of MSC that contains a significant percentage of hematopoietic cells, MSC, EC, and peripheral blood cells, as well as a myriad of growth factors and cytokines. Sophisticated harvesting trocar was developed to guarantee the availability of 30ml to 60ml BMA via a small and cosmetic incision. A centrifuge can be used optionally according to the specific needs of individual clinical situations to separate BMA into stem cell-rich and stem cell-poor portions [211].

BMA can be delivered in various ways. Decades ago, the intravenous infusion of closely matched allogeneic MSC was used to increase bone mass in infants suffering from osteogenesis imperfecta [275]. As for local bone augmentation, BMA can be administered by direct injection or pre-seeding within osteoconductive scaffolds that are commonly synthetic biomimetic constructs, allograft, or xenografts [276 277]. Numerous pioneer groups from dentistry, maxillofacial surgery, and orthopedic surgery have evaluated the potential of this strategy. In addition, several manufactures of FDA-approved synthetic bone substitutes—for example, collagen, IntegraOS (IntegraLifesciences, Inc, Plainsboro, NJ, USA) and Vitoss (Orthovita, Inc, Malvern, PA, USA)—have been advocates for the combined use of BMA [219 278]. However, extensive studies have reported the inconsistent outcomes of using this strategy—some groups have shown improved bone growth while others have had no improvement [219]. Furthermore, the characteristics of the biomaterial substrate to achieve a best outcome in combination with BMA have yet to be defined.

2.2.2.3. Growth factors

Growth factors refer to those proteins or peptides that are secreted by cells and act on target cells with autocrine, paracrine, or endocrine routs. Their short half-life span and slow diffusion in the extracellular matrix inhibit them from acting in distant loci as the hormones of the endocrine system [279]. The spatial distribution and release dynamics of growth factors that contain ECM-binding domains is largely governed by their interactions with extracellular matrix molecules [280]. Growth factors bind specific cell surface receptors and induce biological effects that include cell proliferation, differentiation, matrix secretion, and tissue formation. Since the orchestrated expression of growth factors that are chondrogenic, osteogenic, and angiogenic play indispensable roles in bone development and fracture repair, it is a reasonable to consider

applying these recombinant growth factors for BTE. So far, the study of the application of growth factors for repairing skeletal defects has mainly been limited to the preclinical stage with limited experience in clinical trials[120].

2.2.2.3.1. Candidate molecules

The cytokines and growth factors that dictate the fracture healing cascade are potential candidate molecules for BTE. The most commonly investigated ones are listed below.

Bone morphogenic protein (BMP)

In the 1960s, the existence of an osteoinductive substance in the extracellular matrix of bone was implied by the fact that the implantation of demineralized bone matrix (DBM) in the muscle pouch in rats led to ectopic bone formation [41]. The responsible protein was then identified and named as a bone morphogenic protein (BMP), the sequence of which was later deciphered and led to the mass production of recombinant human BMPs (rhBMPs) for therapeutic use [281 282]. BMPs comprise a group of structurally related proteins that belong to the TGF-β superfamily [52]. They promote the proliferation and differentiation of MSC and play indispensable roles in embryonic organ development, limb formation, and fracture healing [47]. The most studied BMPs are BMP-2, BMP-4, and BMP-7(OP-1), and their potential applications include fracture non-union, open fracture, reconstruction of bone defect, arthrodesis, spinal fusion, distraction osteogenesis, and so on [283]. Only rhBMP-2 and rhBMP-7 have been evaluated for efficacy in randomised, multi-centric, and controlled clinical trials, which found dose-dependent bone formation and reduced healing time due to their administrations [46 284]. To avoid rapid diffuse and sustain local concentration, BMPs have been delivered in association with carriers, such as

collagen sponge, PLA, PLGA, ceramic, allograft, autogenous iliac crest bone graft, and so on [285] [286].

In spite of the benefit of BMPs for bone augmentation, convincing data is still lacking to show that BMPs are superior to autografts with respect to bone repair. In addition, the beneficial effect of BMPs often requires a dosage as high as tens of milligrams [284]. Some researchers have reported the formation of dosage-dependent cysts related to BMP-2 use, which may impair mechanical strength [287]. The local supraphysiological concentration of BMPs raises a concern about their safety with respect to several severe adverse effects such as malignant bone tumor and bone marrow fibrosis [288] [289]. The high demand for BMPs dosage will inevitably incur extra medical expenses, which ultimately increase the financial burdens of patients and society.

Platelet derived growth factor (PDGF)

The PDGF family is comprised of various forms including PDGF-AA, PDGF-BB, PDGF-AB, and PDGF-CC that are released from the granules of aggregated and activated platelets during the coagulation cascade, which become entrapped in developing blood clots [290]. Binding to the surface receptors of the cells of mesenchymal origins, PDGF becomes a chemoattractant to recruit osteogenic progenitor cells [291], mitogen to induce cell proliferation [292], and an angiogenic factor to promote vascularization [293]. In addition, PDGF destabilizes blood vessels purportedly due to the pericytes and smooth muscle cells; therefore, some researchers have proposed that PDGF contributes to fracture healing by using vasculature-pericyte-MSC-osteoblast dynamics [294]. Other proposed mechanisms by which PDGF improves fracture healing include the upregulation of VEGF expression in mural cells, stabilization of newly formed blood

vessels, and the enhancement of osteoblastic differentiation by decreasing the expression of the BMP inhibitory protein gremlin [294].

Researchers have found that PDGF possesses a broad range of wound healing capacities for both soft and hard tissues [295]. As such, rhPDGF-BB has been approved by the FDA for the repair of periodontal defects due to its demonstrated safety and efficacy in inducing a robust regeneration of various tissues including new bone, cementum, and periodontal ligament [296]. Also, rhPDGF was the first FDA approved recombinant protein for the treatment of chronic foot ulcers in diabetic patients [297]. The efficacy of PDGF delivered in various vehicles, such as collagen gel, TCP, and allograft, for healing skeletal defects and fractures are under investigation using preclinical models and clinical trials [294].

Fibroblast growth factor (FGF)

In human FGF family has been recognized to include 22 structurally-related polypeptides that are characterized by their affinity for glycosaminoglycan heparin-binding sites, which have been implicated in wound healing and embryogenesis [298]. FGFs play important roles in mitogenesis and angiogenesis by binding to the cell surface receptor with tyrosine kinase activity and by triggering an intracellular cascade of the MAPK signalling pathway [299 300]. The FGFs related to bone regeneration and fracture healing are FGF1 and 2 as well as FGF4, 8, 19, among which FGF1 (acid FGF) and 2 (basic FGF) are the most widely studied. FGF1 has been recognized as a potent mitogen for chondrocytes [301] while FGF2 has been expressed mainly in osteoblasts and is considered as the most promising member of the FGF family for BTE [302]. FGF2 enhances the proliferation of bone marrow-derived stromal cells and their osteoblastic differentiation while inhibiting the apoptosis of immature osteoblasts [303]. Researchers have

proven the angiogenic potential of FGF2, which is attributed to the induction of smooth muscle differentiation and stabilization of microvasculature structure [304].

Various types of preclinical models have demonstrated that the FGF2 carried by biomaterial vehicles has a capacity for accelerating new bone formation with improved mechanical properties [305 119]. Researchers also have reported a dose dependent effect of rhFGF2 delivered in gelatin hydrogel regarding the healing of fresh tibial fractures in human patients [306]. Other FGF family members, such as FGF9 and FGF18, have started to draw researchers' attention, since they have been reported to improve bone regeneration in preclinical models [307]. However, the biphasic effect of some FGFs on bone formation has raised some concerns: lower doses of FGF2 inhibit bone formation, whereas high doses of FGF2 increase bone formation *in vivo*; brief FGF2/9 treatment enhances osteoblastic differentiation, whereas continuous FGF2/9 treatment inhibits osteoblastic differentiation and mineralization [308 309]. Therefore, the dosage, timing, and duration of FGF treatment needs to be further defined. Caution should be exercised to exclude the possible risk of tumorigenesis and metastasis related to FGF use.

Vascular endothelial growth factor (VEGF)

VEGF is considered to be the most competent angiogenic factor to play a paramount role in bone development, fracture repair, and skeletal homeostasis [57]. So far, at least 7 isoforms of VEGF with the number of amino acid residues ranging from 121 to 206 in humans have been identified due to various splicing combinations [310]. Among them, the most frequent and best-studied are VEGF121, VEGF165, and VEGF189. Due to the similarities of VEGF expression and function in humans and mice, murine models have been used extensively in the study of VEGF [311]. The major murine variants of VEGF are denoted as VEGF120, VEGF164, and

VEGF188, with VEGF 164 being the most dominant in bone [312]. Numerous groups have published promising results for VEGF treatment in various preclinical models of skeletal defects, with reported increases in bone mass and improvements in mechanical structures [50]. However, VEGF needs to be further evaluated for its efficacy and safety in large clinically-relevant animal models before it can be recommended for clinical applications.

Platelet rich plasma and platelet gel

Autologous platelet rich plasma (PRP) is among the newly developed technologies for autologous blood component therapy. In clinical practice, PRP initially reduced hemorrhage and decreased the chances of blood transfusion[313]; in addition, it also improved tissue regeneration and wound healing that has been attributed to the various growth factors contained in the α-granules of platelets, such as TGF-β, PDGF, EGF, VEGF, and FGF [314]. The activation of PRP by the addition of thrombin and Ca^{2+} releases these growth factors and forms a viscous platelet gel that can be injected by using a syringe or transplanted to the local injury site during open surgery [315]. However, conflicting data exists with respect to the efficacy of applying PRP in clinical and preclinical studies, which could be due to the lack of standardization of the protocols used for withdrawing blood, quality control of PRP, the platelet and growth factor count, the activation of PRP, and the approaches used to deliver PRP. Thus, further studies to examine PRP using relevant animal and clinical models are needed to provide more evidence of the outcome of PRP therapy, clarify the risks, and provide clinical guidance before recommending its clinical use.

2.2.2.3.2. Approach

The success of growth factor therapy relies largely on using sufficient concentrations that mimic the biological molecular events that occur during the natural healing processes [316]. The

solution formula delivered through classical infusion is believed to be a disadvantageous strategy for administering growth factors for several reasons. First, the rapid degradation of growth factors caused by denaturation, oxidation, internalization, or proteolysis leads to a half-life lasting only a few minutes; thus, the extremely low concentration of growth factors at the specific tissue level is insufficient to orchestrate the reparative cells in regeneration [120]. To compensate for this short half-life, multiple administrations of growth factors at supra-physiological concentrations could lead to a transient biological response, but large quantities of growth factors may result in severe adverse effects including tumorigenesis [317]. Therefore, it is necessary to develop proper delivery systems for growth factors through which they can be protected from degradation, and the temporospatial control of their accessibility for healing tissues can be realized [120]. Currently, the controlled release of growth factors can be achieved by either biomaterials based vehicles or by *in vivo* production following gene therapy [279].

2.2.2.3.2.1. Biomaterial vehicle

Growth factors have been used by material scientists to enhance biocompatibility and bioactivity as described in previous sections; in addition, biomaterial scaffolds function as carriers for growth factors that allow controlled release. The biomaterials used as delivery matrices for growth factors in experimental and clinical models are mainly polymers and ceramics. The former includes both natural polymers—such as collagen and fibrin, and synthetic polymers, for example, PGA and PLA; the latter category encompasses the bioglass and calcium phosphate-based ceramic preparations, such as HA and TCP [123]. These materials share physical or chemical similarities more or less with ECMs; therefore, they also serve as the carriers for the simultaneously transplanted cells or mediating scaffold for the migrating endogenous cells that are involved in the regeneration process [132 258]. Two strategies have been explored extensively so

to present growth factors through biomaterial vehicles—chemical conjugation and physical encapsulation.

Surface conjugation

Several techniques have been developed to establish the binding between growth factors and biomaterials, which can be non-covalent or covalent [318] [120]. The non-covalent binding, also known as physical adsorption, exploits hydrogen bonding, hydrophobic interactions, or electrostatic charges to immobilize the growth factors into the biomaterials vehicle without changing the molecular formula [319]. Intermediate molecules and small oligopeptides that mimic key functional fragments are sometimes used to link growth factors and material matrices [320]. This approach is exemplified by the heparin-based growth factor delivery system that relies on the innate affinities of growth factors to heparin, which are grafted onto the surface of the material substrate [321]. Numerous studies have found that the VEGF adsorbed on the heparin-tethered-collagen sponge preserves its pharmacological effects and induces angiogenesis [321]. The release kinetics of non-covalently immobilized growth factors not only depends on the strength of association but also is susceptible to the host environment, for example, temperature, pH, and so on [120].

Direct covalent tethering is achieved by connecting the functional groups of the biomaterials with those of growth factors under enzymatic catalyst [322]. Researchers have reported that the multitude of growth factors, including BMP2, VEGF, TGF-β, and EGF, remain competent and capable of inducing specific biological responses after being covalently conjugated to various polymeric and ceramic materials [120]. The pharmacokinetic profile of these covalently tethered growth factors is in sync with the degradation rate of matrices, which usually

is comprised of an initial burst effect and a substrate-dependent secondary release with various half-life times [318]. However, the three dimensional patterning of the growth factors on the matrices is difficult to control; in addition, the specificity of the coupling sites of the conjugated growth factors cannot be selected, which often leads to a loss of bioactivity [120].

The bioactivity and biocompatibility of the growth factors material scaffolds dictate the adhesion of cells to the implanted materials [323] and thus are paramount in potentiating the biological effects of the growth factors. The higher the bioactivity and biocompatibility of the material matrices is, the closer the host cells get to the growth factors incorporated in the matrices. Thus, the cell adhesion oligopeptides that mimic the adhesion moieties of the matrix molecules have been exploited and covalently tethered to the vehicles to enhance the biological responses to the delivered factors [324] [325]. Researchers have found that the adhesion peptide-presenting scaffolds, such as the one that has arginine-glycine-aspartic acid (RGD) tethered, support cellular survival and function, as well as enhancing angiogenesis and wound repair [197].

Bulk loading

The growth factors encapsulated physically *in vitro* by biomaterials can undergo diffusion and pre-programmed release *in vivo* [120]. This approach is a popular alternative to chemical conjugation because of the simplicity of the technique, the controllability of release kinetics through adjustments of the variables of vehicle materials, and the possibility of delivering multiple growth factors in a sequential manner by combining various materials [326] [327]. A wide range of biomaterials including synthetic and natural polymers can be fabricated into vehicles in the form of nanoparticles (1–100nm) /microparticles (1–100μm), nanoporous/macroporous structures, or hydrogels that can be either transplanted or injected.

Diffusion underlies this delivery mechanism of the physical encapsulation system, so the factors that affect this process—such as the surface/volume ratio of microsphere/nanospheres, the porosity of the scaffolds, the material degradation rate, and the polymer molecular weight distribution—can be used to tune the release kinetics of growth factors that contribute to healing tissues [176 258]. The associations between growth factors and vehicle materials are based on the hydrophilic-hydrophilic and hydrophobic-hydrophobic interactions, and manufacturing these physical encapsulation systems necessitates the avoidance of harsh conditions that cause the to-be-encapsulated growth factors to become inactive. The available manufacturing techniques, such as solvent casting/particulate leaching, freeze drying, high internal-phase emulsion, *in-situ* polymerization, and gas forming to name only a few are characterized by their own advantages and limited in other aspects [120]. The strategy of combining different manufacturing methods and materials is often utilized to bypass the drawbacks of individual techniques and to generate more complex patterns for growth factors encapsulation to optimize release kinetics, for example, the porous scaffold incorporating microspheres that contain pre-encapsulated growth factors [326]. This strategy is particularly promising for the sequential delivery of multiple growth factors that simulate natural protein expression during tissue regeneration. The release of VEGF followed by PDGF via this complex delivery vehicle has induced more angiogenesis than the simultaneous release of both of them [328].

To date, the progress that has been made in the development of "smart materials" that release growth factors in response to triggering factors has relied on the changes of local environmental signals or externally applied cues [329]. The moieties contained in the substrate molecules that are able to display physical or chemical transitions in response to certain stimuli—such as pH value, temperature, protease, drugs, ions, light, and magnetic/electric

field—are the key elements for the inducible release of incorporated growth factors [120]. Careful design of triggering systems that are applicable to *in vivo* use is the prerequisite for fast on-off switches and the precise control of the spatiotemporal presentation of growth factors.

2.2.2.3.2.2. Gene therapy

Gene therapy is a promising strategy for the sustained delivery of growth factors, which consists of the insertion of the gene segments encoding certain growth factors into recipients' cells and the persistent production of the recombinant growth factors *in vivo* [330]. The synthesis of growth factors can be short-termed or long-termed depending on the technique used, which, in turn, should be dictated by the needs of the clinical situations. Generally speaking, chronic diseases, such as osteoporosis, require long-term delivery; however, the repair of bone defects, such as fracture non-union, warrants the application of the short-term expression of growth factors [331]. Both *in vivo* and *in vitro* methods have been explored for transferring genetic information into the target cells: the former uses a direct delivery of gene vector to the lesions, whereas the latter transfects or transduces the cultured cells before implantation into the lesions [330]. The gene vector can be viral, such as lentivirus and adenovirus, or non-viral, such as bacterial plasmid [332 333]. Even though gene therapy has been proven to be a reliable and economic technique to realize the sustained production of growth factors in experimental models [334 101], its translation into clinical use for patients requires thorough evaluations of the safety issues, which include but are not limited to the generation of replication competent viruses and immune responses to transgene or viral products [335].

Chapter III: Thesis Hypotheses and Specific Aims

With respect to bone reconstruction, the synthetic bone substitutes engineered by a regenerative medicine strategy could be better options than autogenous and allogeneic bone grafts because their availability is unlimited and pathogen transmission is avoided. Bone marrow- derived mesenchymal stem cells (BM-MSC) are considered to be a plausible osteogenic cue for BTE in that they are competent osteoprogenitors that can be induced to differentiate into osteoblasts and form mineral nodules *in vitro*. The survival of BM-MSC in synthetic bone grafts necessitates sufficient angiogenesis and dictates the success of BTE constructs. My thesis work has explored the modalities of bone reconstruction in preclinical rodent models by an *in vivo* utilization of exogenous BM-MSC and synthetic materials with the addition of an angiogenic factor, VEGF, when indicated. The degradability of the synthetic material used for bone reconstruction governs the requirements for the quality and quantity of regenerated bone with *en bloc* bone formation in case of degradable materials being used and bone appositional growth for osseointegration of non-degradable materials. To comprehensively evaluate the potentials of BM-MSC for bone augmentation in both degradable and non-degradable materials, my hypotheses follow:

Hypothesis I: The transplantation of BM-MSC can improve the osseointegration of an intraosseous implant. To address this hypothesis, specific aims were attained as follows. This part of my thesis work was published in the *Journal of Orthopedic Research* and is included in my thesis as Chapter IV.

Aim 1: Fabrication of Ti implants for *in vivo* use and implant grade Ti inserts for *in vitro* studies

Aim 2: MSC isolation and culture on Ti inserts to analyze the viability and differentiation of MSC on the Ti substrate *in vitro*

Aim 3: Conditioning the irradiation of murine recipients for allogeneic MSC transplantation

Aim 4: Generation of a surgically modified murine model for simultaneous MSC transplantation and intramedullary Ti rod implantation

Aim 5: Development of methodologies for the quantitative MicroCT analysis and comparative histological evaluation for the post-mortem assessment of bone apposition on the Ti implant at postoperative 6 weeks

Hypothesis II: The transplantation of BM-MSC seeded in dense collagen scaffolds with VEGF treatment can promote the healing of large bone defects. To address this hypothesis, specific aims were attained as follows. This part of my thesis work was published in *European Cells & Materials* and is included in my thesis as Chapter V.

Aim 1: Seeding *in vitro* expanded BM-MSC into dense collagen scaffolds

Aim 2: Evaluation of the metabolism and osteoblast differentiation of MSC seeded in the dense collagen scaffolds, as well as the mineralization of collagenous matrices *in vitro* to determine the optimal timing for their *in vivo* use

Aim 3: Generation of a murine model of large bone defects treated with an MSC-seeded dense collagen scaffold prepared as above, and development of a novel surgical approach to deliver a bolus dose of VEGF when indicated

Aim 4: Establishment of standardized MicroCT and histological protocols for the post-mortem evaluation of bone regeneration and neovascularization in the bone defect at post-operative 4 weeks

Hypothesis III: The VEGF tethered in a collagen sponge can improve the repair of critical-sized bone defects. To address this hypothesis, specific aims were attained as follows. This part of my thesis work is included in my thesis as Chapter VI, and will be submitted for publication.

Aim I: Generation of a collagen sponge tethered with VEGF via covalent bonds, and seeded with *in vitro* expanded BM-MSC

Aim II: Development of critical-sized bone defects measured 4mm x 4mm in rat mandibles and treated by the collagen sponge prepared as above

Aim III: Establishment of methodologies for vasculature analysis by MicroCT imaging and immunohistochemistry to evaluate angiogenesis at postoperative 3 weeks and 6 weeks

Aim IV: Standardization of MicroCT and histological analytic protocols for bone regeneration to perform post-mortem assessment at postoperative 8 weeks

IV. MSC transplantation to promote osseointegration

Titanium (Ti) is the metal material most commonly utilized for intraosseous implants partially because of its capacity of being osseointegrated in the host bone. Several technologies can increase the osteoconductivity and osteoinductivity of Ti implant by modifying the chemical composition and surface topography in order to enhance the bone apposition on the implant surface. However, the mechanism that increases bone appositional growth around the implant by applying osteogenic cues, i.e. osteogenic cells, has not been reported yet. This chapter describes a novel approach that uses BM-MSC transplantation to improve osseointegration of an intramedullary Ti implant.

The cell based approach to promote osseointegration of Ti implants is proposed by us to be useful in the recipients where the innate bone regeneration is impaired and the peri-implant new bone formation relying on the endogenous healing mechanism is insufficient. In the context of declined availability of bone forming cells, such as aging, post-chemotherapy, or post-radiation, this approach is thought to be particularly helpful in restoring the osteogenitor population and increasing the availability of osteogenic cells for osseointegration. To simulate the specific clinical scenario we used FGFR3-/- mice whose skeletal phenotype has been characterized by our lab previously. FGFR3-/- mice display a reduced cortical bone thickness and a defective trabecular bone mineralization. Furthermore, these FGFR3-/- mice underwent sub-lethal irradiation in their hind limbs before allogeneic BM-MSC transplantation treatment in order to ablate the endogenous BM-MSC and further impair the endogenous healing capacities. Of note, conditioning irradiation is also often employed before HSC transplantation to decrease the immune rejections and improve the engraftment of transplanted cells.

I established the experimental protocols, conducted the bulk of the experiments, and performed data collection and analysis as well as paper drafting. Drs. Janet Henderson and Edward Harvey provided essential supervision throughout the project and edited my manuscripts extensively for publishing. Dr. Jan Seuntjens, as our collaborator, helped me to perform irradiation conditioning of murine recipients for stem cell transplantation. Gabriel N. Kaufman did the growth and differentiation assay. Ailian Li and Huifen Wang provided technical assistance in histological analysis and animal maintenance, respectively.

Mesenchymal Stem Cell Transplantation to Promote Bone Healing

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Running title: bone regeneration and osseointergation

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ABSTRACT

An overall decline in the availability of osteogenic precursor cells and growth factors in the bone marrow microenvironment have been associated with impaired bone formation and osteopenia in humans. The objective of the current study was to determine if transplantation of mesenchymal stromal cells (MSC) from a healthy, young donor mouse into an osteopenic recipient mouse could enhance osseointegration of a femoral implant. MSC harvested from normal young adult mice differentiated into bone forming osteoblasts when cultured on implant grade titanium surfaces *in vitro* and promoted bone formation around titanium-coated rods implanted in the femoral canal of osteopenic recipient mice. Micro computed tomographic imaging and histological analyses showed more, better quality, bone in the femur that received the MSC transplant compared with the contra-lateral control femur that received carrier alone. These results provide pre-clinical evidence that MSC transplantation promotes peri-implant bone regeneration and suggest the approach could be used in a clinical setting to enhance bone regeneration and healing in patients with poor quality bone.

KEYWORDS

MSC transplant; conditioning irradiation; osteopenic mouse; intra-femoral implant; micro CT

INTRODUCTION

Age-related changes in the bone marrow microenvironment result in a decrease in the number and decline in the function of the mesenchymal stromal cells (MSC) that differentiate into bone forming osteoblasts [336]. These changes impact negatively on bone ingrowth that is required for the rapid and stable fixation of prostheses, as well as on the surgical repair of fractures [337]. The replacement of diseased joints with prosthetic devices is currently the most common major reconstructive orthopaedic procedure performed worldwide. It remains the only effective solution for the relief of pain and restoration of joint function to millions of people with advanced arthritis [338]. In North America total hip replacements performed on reasonably young, active recipients in the past two decades have used implants with porous metallic or ceramic surfaces that rely on host bone in-growth or "osseointegration" for early fixation and long term stability [339]. Modifications to the femoral stem to simulate the texture of bone are ineffective in an environment where there is an underlying deficiency in MSC or lack of anabolic factors to induce them to differentiate down the osteogenic lineage. The MSC pool can also be depleted in younger individuals as a result of treatment for cancer or graft versus host disease [340]. The majority of joint replacements performed under these circumstances use acrylic cement to immobilize the implant stem in the femoral canal [341]. Complications that arise from the use of this cement include bone cell toxicity and an inflammatory response to particulate matter, which result in aseptic loosening and mechanical failure of the implant [342].

A reduction in endogenous MSC due to advancing age or an underlying medical condition will also compromise fracture healing. Delayed fracture healing following orthopedic trauma in the older patient leads to mal-union or non-union in up to 25% of cases and is

exacerbated in those who have underlying medical conditions [343]. More than a decade ago it was estimated that the direct healthcare costs of treating a non-union were five times those of treating a simple hip fracture [344]. Despite significant advances in the surgical management of fractures in poor quality bone the costs associated with clinical complications resulting from delayed fracture healing continue to rise along with the mean age of the population [345]. Therapeutic options currently available to augment bone regeneration during fracture healing are restricted to the use of devitalised allografts supplemented with vascularised fibular grafts [346] or bone morphogenetic proteins (BMP) to stimulate recruitment and differentiation of osteogenic cells [337].

The objective of the current study was to obtain proof-of-concept that transplantation of MSC from young healthy donor mice could enhance implant osseointegration in mutant littermates that exhibit skeletal defects similar to those seen in ageing human bone. Fibroblast growth factor receptor 3 (*fgfr3*) encodes one of four high affinity receptors for a family of 22 related FGF ligands [299]. Alternative splicing and temporal-spatial restriction of their expression patterns, as well as that of co-receptors and ligands, account for the specificity of their biological function. Detailed analysis of the skeletons of young adult FGFR3$^{-/-}$ mice revealed osteopenia that was due in large part to defective MSC differentiation, identifying them as a model to examine MSC transplantation as a mechanism to enhance bone formation [347].

MATERIALS and METHODS

MSC isolation and culture on implant grade titanium

All mice used for i*n vivo* and *in vitro* studies were obtained from a C3H breeding colony maintained by our lab at McGill University for more than 10 years and derived from founders from the colony maintained by D.M. Ornitz at Washington University. Whole bone marrow was extracted from the long bones of three 4-6 month old $FGFR3^{+/+}$ mice and the MSC isolated by adherence to tissue culture plastic (TCP) as described previously [347] [348]. Discs measuring 21 mm in diameter and 1.5 mm thick were fabricated by Changzhou Kanghui Joint Implants Company (China) from implant grade titanium and polished to generate a surface with an average roughness comparable to tissue culture plastic. Prior to use, all discs were cleaned in 100% ethanol, passivated in 30% nitric acid at room temperature for 30 minutes, rinsed repeatedly in distilled water and steam sterilized. MSC were plated at $5x10^4$ cells per well on titanium inserts, or directly on TCP, in 12-well plates. Replicate cultures were removed at the indicated times, fixed with 4% paraformaldehyde (PF) and stained with alkaline phosphatase (ALP) as an early differentiation marker or with 2 µg/mL Hoechst 33342, rhodamine phalloidin and calcein to evaluate terminal differentiation [349]. Metabolic activity and ALP enzyme activity (Table 1) were measured essentially as described [350].

RNA extraction and RT-PCR analysis

Total RNA was extracted at the indicated time points from four replicate cultures grown on plastic or titanium surfaces in parallel with those used for light and fluorescence microscopy. Reverse transcription and semi quantitative PCR analyses were performed essentially as described previously [347] [348] to examine the expression of recognized markers of osteoblast

differentiation. PCR products were obtained in the linear range of amplification and quantified by scanning densitometry with Image J software (NIH). GAPDH was used as an internal control to normalize the signal from genes of interest.

Micro-fabrication of titanium coated implants for *in vivo* use

Semi-rigid 0.4 mm diameter nylon line was cleaned by sonication for 30 minutes in a mixture of equal volumes Renuzyme (Getinge, NY, USA) and Liqui-Nox (Alconox, NJ, USA) at 50° C then with 2% NaOH at 21° C before rinsing thoroughly with distilled water. A 200 nm thick layer of commercial grade titanium was then deposited evenly onto the line using physical vapour deposition at the McGill Institute for Advanced Materials (http://www.mcgill.ca/miam). 10mm lengths stored in 70% ethanol were rinsed several times with sterile PBS before implanting in the femoral canal.

Conditioning irradiation of recipient FGFR3$^{-/-}$ mice

All animal procedures were performed according to protocols approved by the McGill University Animal Care Committee in compliance with the guidelines of the Canadian Council on Animal Care. Eight male FGFR3$^{-/-}$ mice aged 4-6 months were sedated and positioned in a custom built box with a lead shield covering the upper body as shown in Fig 4.3. An accurately calibrated narrow-collimated 6 MV ionizing radiation beam, produced by a Clinac 6Ex linear accelerator (Varian, Palo Alto, CA), delivered 13.5Gy to the lower body. The absorbed dose of irradiation to the lower body was determined using procedures outlined by Seuntjens et al [351]. Bone marrow was harvested from 3 FGFR3$^{+/+}$ mice, 3 irradiated FGFR3$^{-/-}$ mice and 3 non-irradiated FGFR3$^{-/-}$ mice and the adherent MSC left to adhere for 6 days before counting to determine post-irradiation survival (D0). The FGFR3$^{+/+}$ and irradiated FGFR3$^{-/-}$ MSC were then

re-plated at a density of $2x10^4$ cells per well in 24 well dishes to compare their proliferation rates after 3, 6 and 9 days in culture.

MSC transplant and femoral implant in FGFR3$^{-/-}$ mice

The eight irradiated FGFR3$^{-/-}$ recipient mice were allowed to recover for 48 hours before undergoing surgery to deliver MSC from FGFR3$^{+/+}$ donor mice, along with the bio-compatible implants, into the femoral canal. MSC were isolated from femoral bone marrow of eight normal 4-6 month old FGFR3$^{+/+}$ mice, from the same colony as those used in the *in vitro* study, and re-suspended at a concentration of 10^5/10µl sterile type I collagen solution 1mg/ml (Invitrogen) pH 7.4. At 48 hours post irradiation anaesthesia was induced and maintained with inhaled vaporized 2% isoflurane before shaving the skin and exposing the trochanter and proximal femur bilaterally through 15 mm skin incisions. The MSC suspended in collagen solution were drawn up into a 100µl Hamilton syringe and the needle inserted into the femoral canal via the piriformis fossa medial to the greater trochanter. 10µl of cell suspension was injected slowly into the femoral canal, immediately prior to inserting a titanium-coated implant. MSC in 10 µl of collagen were injected into the RIGHT femoral canal and 10 µl of collagen solution alone as control into the LEFT femoral canal. The biocompatible titanium coated implants measuring 0.4mm x 10mm were then fed through a 25G needle into the femoral canal before closing the incision. All mice received buprenorphine for 2 days as post-operative analgesia and were allowed free access to food and water for 6 weeks, when they were euthanized and their femurs harvested for analysis.

Micro CT and histological analysis of peri-implant tissue

Femurs were dissected free of soft tissue, fixed overnight with 4% paraformaldehyde, washed and stored at 4^o centigrade prior to conducting micro CT scans on a Skyscan 1172 (Kontich, Belgium) instrument equipped with an x-ray source of 10 w/100 kV and a 10 mega-

pixel camera [347] [348]. NRecon and CTAn software enabled scanning and 3D reconstruction of specimens at a resolution of 5 μm for comparison with histological sections cut at the same thickness. A peri-implant cylinder measuring 0.05 mm x 2 mm spanning the lesser trochanter (Fig 4.4C) was identified as the region of interest for quantification of newly formed bone. The 3D bone morphometric parameters used in this paper include bone volume per tissue volume (BT/TV, propotion of defined volume of interest occupied bone), bone mineral density (BMD, volumetric density of calcium hydroxyappetite), trabecular bone pattern factor (TbPf, index of bone connectivity), structural model index (SMI, indicator of relative prevalence of rod and plates in 3D structure), and trabecular thickness (TbTh, average local thickness of bone structure). After micro CT analysis the femurs were dehydrated in graded alcohols and embedded at low temperature in plastic for histological analyses. Serial 5 micron sections of un-decalcified bone were stained with Von Kossa/Toluidine Blue (mineral content), ALP (osteoblasts) or TRAP (osteoclasts) as described previously [347] [348].

Statistical analyses

In vivo data is representative of that obtained from 8 $FGFR3^{-/-}$ mice and *in vitro* assays were performed in 3-4 replicate wells on a minimum of 3 biological replicates. SPSS (IBM) was used to perform a global analysis of variance (ANOVA) with the parameter as the dependent variable and the surface type as the independent variable. Directionality was determined by the sign of the mean difference. Quantitative data for the *in vivo* experiments was expressed as the mean ± SD, and statistical comparisons between femurs made using a paired Student's *t*-test. A probability of $P < 0.05$ was considered to be significantly different.

RESULTS

The primary objective of this study was to determine if transplantation of MSC from young healthy donor FGFR3$^{+/+}$ mice could enhance bone formation on, and around, a biocompatible titanium coated implant in the femoral canal of osteopenic FGFR3$^{-/-}$ mice. Fig 4.1 describes the overall Experimental Design with the *in vivo* and *in vitro* components.

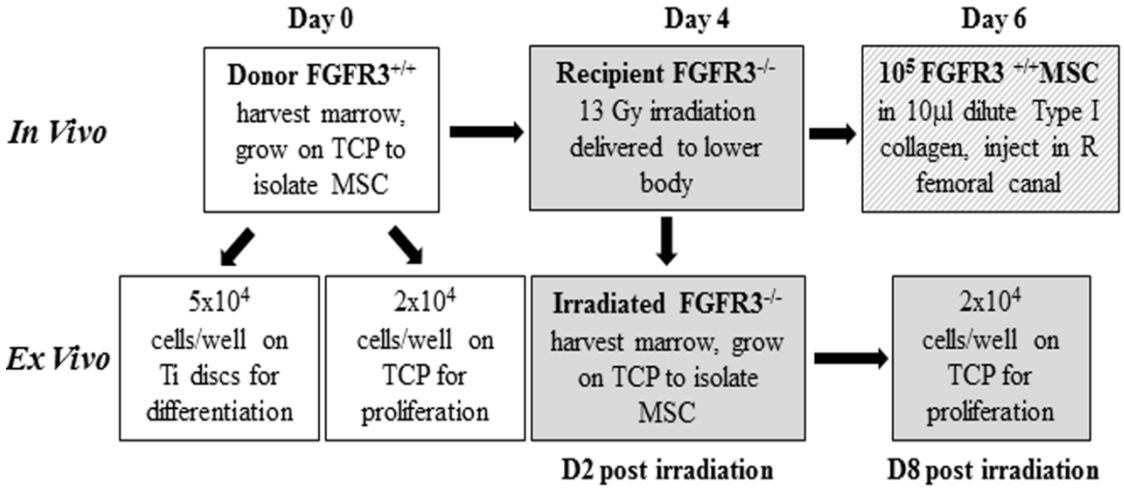

Figure 4.1 Experimental design: Mesenchymal stromal cells (MSC) were isolated by adherence to tissue culture plastic (TCP) from whole bone marrow harvested on Day 0 from FGFR3$^{+/+}$ mice. On Day 6, at 80% confluence, the MSC were trypsinized and used in one of the following three experiments: 1) 5 x 10^4 cells plated on titanium (Ti) discs in 12 well dishes for differentiation studies as shown in Fig 4.2 or 2) 10^5 cells diluted in 10µl dilute Type I collagen and injected into the right (R) femoral canal of FGFR3$^{-/-}$ recipient mice, irradiated on Day 4, as shown in Fig 4.4 or 3) 2x10^4 cells/well plated on tissue culture plastic (TCP) in 24 well dishes for proliferation studies in comparison with irradiated FGFR3$^{-/-}$ MSC as shown in Fig 4.3.

In vitro growth and differentiation of MSC on titanium surfaces

The response of donor MSC isolated from FGFR3$^{+/+}$ marrow to implant grade titanium was first characterized *in vitro.* Fig 4.2 shows that MSC isolated by adherence to tissue culture plastic and plated on titanium formed characteristic condensations that stained positive for ALP (A and B) and supported mineral deposition (D). The quantitative data shown in Table 4.1 indicates the time-dependent maturation of cultures grown on titanium was similar to that of MSC grown on TCP. Cell differentiation, as evidenced by *in situ* ALP staining, increased over time while ALP enzyme activity declined at Day 10 concomitant with mineral deposition. Metabolism increased until Day 10 and then slowed down as the cells reached the end of their lifespan once the matrix mineralized. RT-PCR analysis of RNA harvested at the same time points showed similar expression patterns for recognized markers of osteoblast differentiation, when normalised to GAPDH expression, in MSC grown on TCP or on titanium discs (data not shown).

Table 4.1 *In vitro* growth and differentiation of FGFR3$^{+/+}$ MSC on titanium discs

Assay	Units	Surface	Day 6	Day 10	Day 17
Hoechst 33342 (growth)	Units x 10^{-3}	TCP*	2.79 ± 0.72	10.39 ± 1.64	18.56 ± 2.26
		Titanium	16.20 ± 3.01	16.14 ± 2.41	22.0 ± 1.04
In situ ALP differentiation	Area cm^2	TCP**	0.01 ± 0.01	0.74 ± 0.34	2.87 ± 0.28
		Titanium	0.05 ± 0.01	0.27 ± 0.06	0.62 ± 0.26
ALP activity	nmol/min/mg	Titanium	1.94 ± 0.29	0.62 ± 0.39	0.67 ± 0.27
MTT activity (metabolism)	OD492	Titanium	0.42 ± 0.10	0.84 ± 0.11	0.95 ± 0.25

* P > 0.0001, Global ANOVA, Titanium versus TCP over all time points. ** P = 0.0183, Global ANOVA, Titanium versus TCP over all time points. TC = tissue culture; ALP = alkaline phosphatase.

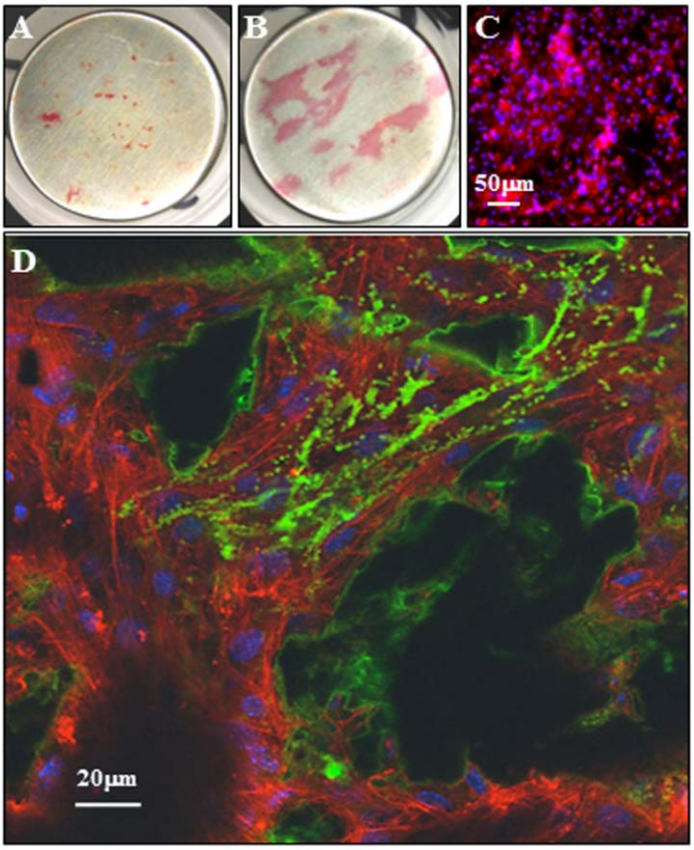

Figure 4.2 Maturation of MSC *in vitro* on implant grade titanium: MSC plated on Ti discs as described in Fig 4.1 were harvested after 10 (A) and 17 (B) days of culture for alkaline phosphatase (ALP) and after 17 days for fluorescence microscopy (C and D). ALP increased over time and correlated with condensation of MSC into nodules, where nuclei stained blue and the cytoskeleton pink (C) or red (D). Cultures stained after 17 days with calcein showed green mineral deposits (D). Data is representative of three independent experiments conducted on MSC harvested from three different mice. Scale bars represent 50 microns (C) and 20 microns (D).

Impact of FGFR3$^{+/+}$ MSC transplant on peri-implant bone growth in FGFR3$^{-/-}$ mice

In previous work we showed that osteopenia in young adult FGFR3$^{-/-}$ mice was due in large part to aberrant maturation of MSC into osteoblasts [347]. In the current work we depleted endogenous MSC in FGFR3$^{-/-}$ mice using sub-lethal irradiation to condition the mice for transplant. Fig 4.3 shows the apparatus used to shield the upper body while 13.5Gy of photon irradiation was delivered to the hind limbs (A). This dose of radiation reduced the adherent MSC population by 80% (B) compared with that from age-matched non-irradiated FGFR3$^{-/-}$ mice (Irradiated: $3.35 \pm 1.05 \times 10^5$ compared to Non-irradiated: $18.08 \pm 4.92 \times 10^5$). When equal numbers of irradiated FGFR3$^{-/-}$ and control FGFR3$^{+/+}$ cells were re-plated on TCP there were 6 fold fewer irradiated FGFR3$^{-/-}$ cells compared with non-irradiated FGFR3$^{+/+}$ cells at Day 9 (C) of culture, indicating a severe impairment in their replication capacity.

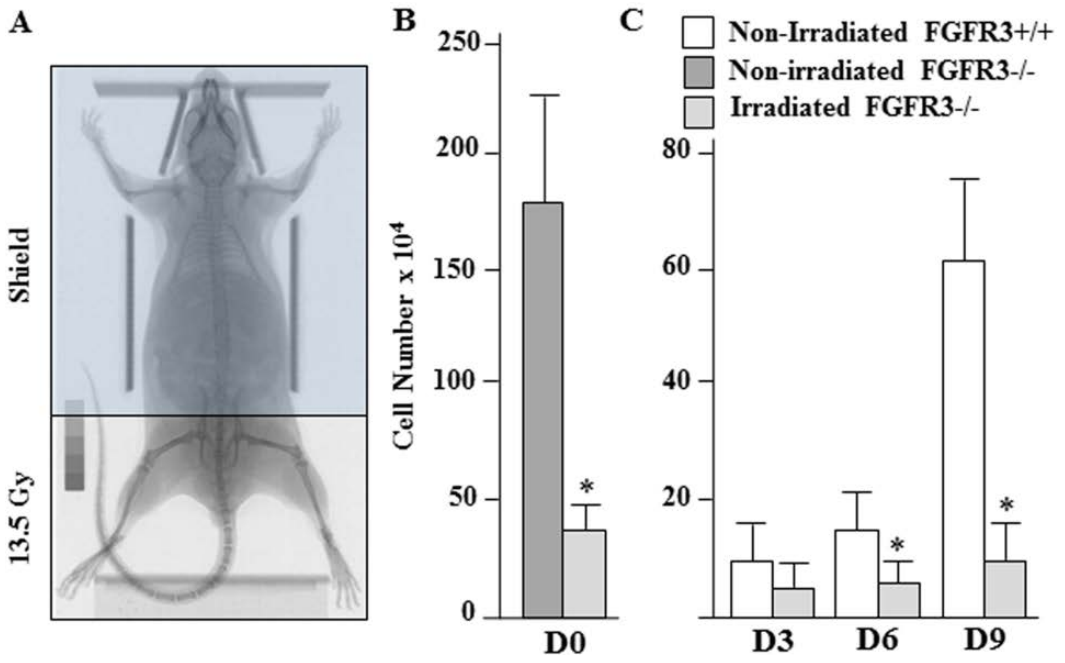

Figure 4.3 Post-irradiation survival and proliferation of MSC *in vitro*: FGFR3$^{-/-}$ recipient mice aged 4-5 months were sedated and positioned in a custom built box with the upper body shielded while the hind limbs received 13.5 Gy of conditioning ionizing irradiation (A). MSC isolated from whole bone marrow of FGFR3$^{+/+}$, FGFR3$^{-/-}$ and irradiated FGFR3$^{-/-}$ mice were left to adhere for 6 days before determining post-irradiation survival (D0) and the FGFR3$^{+/+}$ and irradiated FGFR3$^{-/-}$ MSC re-plated at 2 x 10^4 cells per well in 24 well plates for quantification of cell proliferation at the indicated time points. Irradiation reduced the population of MSC by 80% (B) and impaired the proliferation of the remaining MSC compared with age matched non-irradiated FGFR3$^{+/+}$ mice (C). Data is representative of experiments performed in triplicate. * Significantly different $P < 0.05$.

Bone growth around intra-femoral implants was quantified using micro CT 6 weeks after transplantation of 10^5 MSC from $FGFR3^{+/+}$ donor mice into the right femoral canal of irradiated $FGFR3^{-/-}$ recipient mice. Fig 4.4 shows the location of the implants in situ (A and B) and the 2 mm x 0.05 mm region of interest (B and C), which is shown as a light grey area around the darker rod depicting the implant in the schematic in Fig 4.4C. Significantly more peri-implant bone was seen in the RIGHT femur that received the MSC transplant than the LEFT femur that received collagen carrier as a control. The quantitative data from eight replicates, shown in Table 4.2, revealed a significant increase in bone volume per tissue volume (BV/TV) and a reduction in trabecular pattern factor (TbPf), which is derived from the number, orientation and spacing of trabeculae, in the RIGHT compared with the LEFT femora.

Table 4.2 Quantitative micro CT analysis of bone quality

$FGFR3^{-/-}$		**BV/TV* (%)**	**TbTh (μ)**	**TbPf (1/μ)**	**BMD (g/ml)**	**SMI**
Collagen	Mean ± SD	3.02 ± 1.77	39.1 ± 3.6	35.1 ± 7.4	0.14±0.03	2.16 ± 0.17
	Range	0.99 - 5.90	36.5 - 47.1	21.8 - 47.0	0.11-0.21	1.91 - 2.38
Collagen+MSC	Mean ± SD	6.23 ± 3.93	42.7 ± 4.4	27.6 ± 6.2	0.17±0.06	1.97 ± 0.32
	Range	1.81 - 12.69	37.6 - 48.6	18.8 - 38.3	0.11-0.28	1.52 - 2.47
	P value	0.04	NS	0.01	NS	NS

BV/TV, bone volume per tissue volume; TbTh, trabecular thickness; TbPf, trabecular pattern factor; BMD, bone mineral density; and SMI, structure model index. * $P < 0.04$ and ** $P < 0.01$, Student's t-test collagen alone versus Collagen + MSC

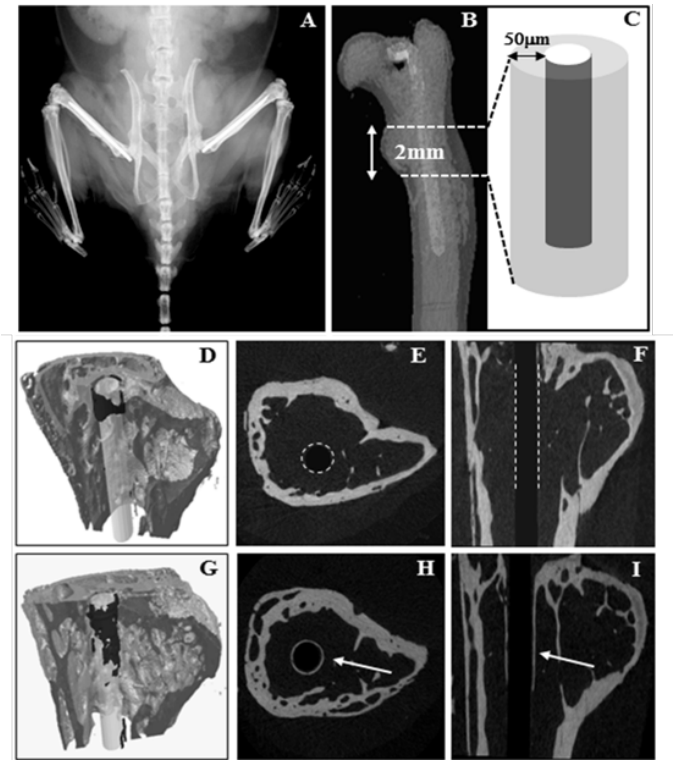

Figure 4.4 Peri-implant bone regeneration after MSC transplant *in vivo*

: On Day 2 post-irradiation recipient FGFR3$^{-/-}$ mice were anesthetized for MSC transplant and implant surgery. MSC isolated as described in Fig 4.1 from 4-6 month old male FGFR3$^{+/+}$ donor mice were transplanted into the RIGHT femoral canal at a concentration of 10^5 /10 µL dilute sterile collagen, along with a biocompatible Ti-coated implant. The LEFT femur received 10 µL dilute collagen with the biocompatible implant (A). Femora were harvested from mice euthanized at 6 weeks post-op, fixed in 4% paraformaldehyde and scanned on a Skyscan 1172 micro CT instrument. A cylinder of tissue 2mm x 50 µm surrounding the Ti-coated implant at the level of the lesser trochanter (B and C) was analyzed for new bone formation. 3D reconstructions show less bone around the implant in the LEFT (D) compared with RIGHT (G) femur. Dotted lines (E, F) on 2D sections show the implant position in the LEFT femur (E, F) and arrows point to peri-implant bone (H, I) in the RIGHT femur. Data is representative of that obtained from eight mice.

Representative results from histological analysis of thin transverse sections of plastic embedded bone, shown in Fig 4.5, confirmed the presence of more peri-implant bone, osteoblast and osteoclast activity in the femora that received the MSC transplant compared with those that received collagen alone.

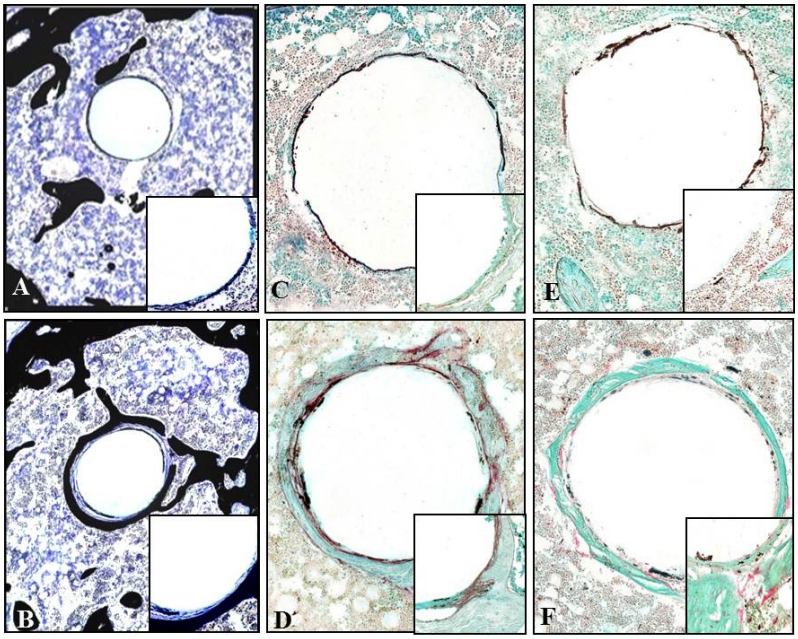

Figure 4.5 Histological analysis of peri-implant bone: After CT analyses the LEFT (A,C,E) and RIGHT (B,D,F) femurs were left undecalcified and embedded in polymethylmethacrylate at low temperature to preserve enzyme activity. Serial 5µm sections were stained with von Kossa and toluidine blue to identify mineral and soft tissue (A,B), alkaline phosphatase (ALP) to identify osteoblasts (C,D) and tartrate resistant acid phosphatase (TRAP) to identify osteoclasts (E,F). More peri-implant bone, accompanied by ALP and TRAP activity, were seen only in the RIGHT MSC treated femora. Magnification x10 through the microscope and x10 objective (A,B) x20 objective (C-F) and x40 objective for all insets. Data is representative of that obtained from six mice.

DISCUSSION

We used the FGFR3$^{-/-}$ mouse with osteopenia and impaired bone growth as a model to study the effect of MSC transplantation on femoral implant osseointegration. In previous work it was shown that targeted disruption of *fgfr3* in mice on a mixed C57BL6J background resulted in skeletal overgrowth *in utero* that continued into the post natal period [352]. When the mutation was bred onto the C3H background longevity was increased allowing for detailed analysis of the adult skeleton [347]. Cortical thickness was reduced in the femoral diaphysis due to sub-periosteal fibrous tissue formation and trabecular bone was lined with cuboidal osteoblasts adjacent to thick osteoid seams. MSC isolated from femoral bone marrow harvested from the FGFR3$^{-/-}$ mice also exhibited impaired differentiation compared with those harvested from FGFR3$^{+/+}$ mice , as evidenced by altered expression of differentiation markers such as osteopontin and osteocalcin [347]. In the current work, Fig 4.2 and Table 4.1 show that MSC from wild type donor mice grow equally well on implant grade titanium discs as on TCP, and differentiate into osteoblasts that form mineralized nodules within 3 weeks.

Targeted high dose irradiation is used clinically prior to an allogeneic stem cell transplant to reduce the risk of rejection and also, by killing endogenous cells, to generate physical space for the transplanted cell population to expand [353]. In a similar manner, 6 MV photon radiation doses of 13.5 Gy were delivered to the hind limbs of FGFR3$^{-/-}$ mice as “conditioning” for an FGFR3$^{+/+}$ MSC transplant to replace the defective endogenous cells (Fig 4.3). After 6 weeks of unrestricted weight bearing activity there was significantly more bone around the implant in the femur that received the MSC transplant than in the contralateral femur that received collagen carrier alone (Fig 4.4). The fact that each animal acted as its own internal control argues against any systemic response to the irradiation or to the intra-osseous MSC transplantation contributing

directly to the unilateral increase in peri-implant bone formation. It was interesting to note that there was no evidence of excessive bone turnover or marrow fibrosis in the control femur, as might be anticipated following irradiation and mechanical reaming of the intra-medullary canal to accommodate the implant (Fig 4.5).

The absence of peri-implant fibrosis was particularly notable as it is proposed to be the end product of a chronic inflammatory response to a foreign body, which poses a major barrier to the integration and biological performance of medical devices including prostheses, implantable biosensors and drug delivery devices. The fibrous response to implanted materials involves binding of fibronectin to integrins on the cell surface [354]. In our previous work that examined integrin expression and function in skeletal cells it was shown that both were disrupted in $FGFR3^{-/-}$ MSC grown *in vitro* [355] and contributed to a fibrous peri-implant response in $FGFR3^{-/-}$ mice *in vivo* in the absence of conditioning irradiation (unpublished data). The absence of fibrous tissue in the current work implies that targeted irradiation of the hind limbs provided sufficient conditioning to suppress inflammation following surgical reaming and to allow engraftment of the donor MSC. The limited bone repair in the control femur could have resulted from migration of the transplanted cells, as has been suggested for non-human primate models [356].

Given the increased quantity and improved quality of the peri-implant bone (Table 4.2) it is predicted that the transplanted $FGFR3^{+/+}$ MSC differentiated into normal osteoblasts *in vivo* that deposited and mineralized a peri-implant bone matrix. Additional studies that measure inflammatory cytokines and the expression pattern of integrins on the surface of endogenous and transplanted MSC are needed to clarify the molecular mechanisms underlying bone regeneration in this implant osseointegration model. The outcome of this study provides proof-of-concept that direct MSC transplantation into the intra-femoral space at the time of implant surgery can

promote new bone formation and enhance biological fixation of the implant in a host with poor bone quality.

CONCLUSION

Direct intra-operative transplantation of allogeneic MSC into femoral canal stimulated bone formation around a titanium-coated femoral implant in FGFR3-/- mice with endogenous MSC ablated by sublethal irradiation. The results of this pre-clinical study suggest that MSC transplantation could be used in the clinical setting to promote osseointegration of titanium hardware in host with impaired bone quality and regeneration capacity.

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Chapter V: MSC Seeded in a Collagen Scaffold Combined With VEGF Treatment to Promote the Healing of Large Bone Defects

Biodegradable scaffolds are gaining more popularity for bone defect repair because they can lead to the formation of new bone that is indistinguishable from the original host bone, and they avoid the complications related to permanently residing foreign materials, such as infection, inferior mechanical strength, and potential toxicity to name only a few. Type I collagen, the main structural protein of osteoid in the extracellular bone matrix, has garnered enormous attention as a natural polymeric material for bone tissue engineering because of its conserved sequence across species, low immunogenicity, and innate osteoconductivity.

Among the numerous researchers who have studied Type I collagen, Prof. R. A. Brown at University College, London developed a dense collagen scaffold by the plastic compression of hydrated collagen gel. During this process, interstitial fluid is rapidly expulsed from hydrated collagen gel to generate a dense collagen scaffold with useful mechanical properties and ultrastructure. By suspending MSC in a Type I collagen solution that later undergoes gelation and plastic compression, MSC can be encased in collagen scaffolds homogenously at different seeding densities. Prof. S. Nazhat has shown that MSC seeded within collagen gel survived plastic compression during the fabrication of the dense collagen scaffold and exhibited superior *in vitro* osteoblastic differentiation compared to those MSC seeded in uncompressed and hydrated gels. This effect can be attributed to the extracellular environment of the gel that is similar to the physiological conditions that facilitate osteoblast differentiation. Also, the cells seeded inside the dense collagen gel have a greater accessibility to nutrients and oxygen compared to those in the hydrated gel. From a surgical point of view, the cell delivery efficiency

in a certain volume of scaffold is increased by approximately 100 times because the plastic compression expels around 98% of the fluid of the hydrated collagen gel and decreases its volume to about 1/100 of its original size. With their ability to form "bone nodules" *in vitro*, the MSC seeded in this dense collagen scaffold appear to be promising for bone defect repair once delivered *in vivo*.

This chapter describes my thesis work that focused on the application of the BM-MSC delivered in the degradable dense collagen scaffold, as mentioned above, to expedite *en bloc* bone formation in a clinically relevant murine model. As angiogenesis and osteogenesis are closely coupled during osteogenesis, we also examined the role of a locally administrated VEGF in the process of large-scaled bone defect healing, as well as the synergistic effect of transplanted MSC and VEGF treatment.

I established the experimental protocols, conducted the bulk of the experiments, and performed data collection and analysis, as well as drafting the paper. Drs. Janet Henderson and Edward Harvey provided essential supervision throughout the project and edited my manuscripts extensively for publishing. Brian Chen, Mardonn Chua, Alison Butler, and Fan Jiang contributed to my work by assisting in cell culture, qPCR, micro computed tomography (microCT) scanning, and histological processing under my supervision. Yicong Liu verified the validity of the statistical analysis. Ailian Li and Huifen Wang provided technical assistance for the histological analysis and animal maintenance, respectively.

MSC-seeded dense collagen scaffolds with a bolus dose of VEGF promote healing of large bone defect

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Running title: *Bone tissue engineering*

Keywords: Bone repair; collagen scaffold; mesenchymal stromal cells; vascular endothelial growth factor

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ABSTRACT

The functional repair of large skeletal defects remains a significant challenge to orthopaedic surgeons due to the lack of effective strategies to promote bone regeneration, particularly in the elderly. This study investigated the potential use of bone marrow derived mesenchymal stromal cells (MSC) in a dense collagen scaffold with a bolus dose of vascular endothelial growth factor (VEGF) to repair a defect in the femoral diaphysis of mice. MSC isolated from bone marrow of 4 month old donor mice were seeded in type I collagen gels that were then compressed to form scaffolds with a fibrillar density similar to osteoid. The cells remained metabolically active in scaffolds incubated *in vitro* for up to 15 days and differentiated into osteoblasts that deposited calcium-phosphate mineral into the scaffold, which was quantified using micro computed tomographic (microCT) imaging. When implanted in a 1 mm x 3 mm unicortical defect the MSC-loaded scaffolds were rapidly mineralized and integrated into host bone with administration of 10 ng of recombinant vascular endothelial growth factor (VEGF) injected into the femoral canal at 4 days postoperative. Empty scaffolds and MSC-seeded scaffolds implanted in defects that did not receive a bolus dose of VEGF did not mineralize or integrate with native bone. The approach with MSC, hydrogels and a biologic factor already approved for human use warrants further pre-clinical investigation with a large animal model.

INTRODUCTION

Traumatic injury and excision of infected or neoplastic tissue all result in large defects that will not heal spontaneously, particularly in older individuals, and require an effective strategy for assisted repair to restore skeletal integrity and function [1]. The surgical reconstruction of large defects in bone requires an osteoinductive material to promote bone regeneration in the gap [357]. Autogenous bone grafts have been considered the gold standard for assisted repair under these circumstances due to their superior osteoinductive and osteoconductive properties. They are, however, limited in supply and lead to donor site morbidities that include pain and infection [281]. Alternatively, de-vitalised allograft bone from cadaveric sources is more plentiful but has poor osteogenic capacity and carries the risk of acquired pathogens such as HIV or HBV [358]. Bone tissue engineering strategies using synthetic scaffolds, cells and soluble molecules may offer a better alternative to the use of human bone grafts to decrease morbidity and increase the potential for repair of large skeletal defects [20].

Bone is a nano-composite material with a 3D hierarchical structure that is composed of calcium phosphate mineral in a collagen matrix. Strategies that have been developed to overcome deficiencies in endogenous repair mechanisms involve the use of “smart scaffolds” as a delivery vehicle for cells and the growth factors they require for osteogenic differentiation. In this context it should be noted that the use of collagen sponges soaked with bone morphogenetic proteins (BMP, eg Medtronic INFUSE) to augment healing of large defects is being re-considered, due in part to questionable efficacy in clinical trials [289 359]. Furthermore, serious side effects such as osteosarcoma, marrow fibrosis and ectopic bone formation have been associated with the use of recombinant BMP. A variety of synthetic materials including degradable polymers, bioceramics (hydroxyapatite) and bioactive glass have also been developed for bone

tissue engineering. Although these materials are approved for clinical use and are easy to fabricate with reproducible structure and mechanical properties, their early promise as temporary scaffolds for bone repair has not materialised. This is due primarily to their poor degradation property and replacement over time with mechanically sound and biologically functional bone. Alternative strategies under investigation involve replication of the hierarchical, porous structure of cancellous bone, with its nano- and meso-scale features, in synthetic scaffolds that enable the attachment and differentiation of bone forming cells and their precursors. Examples of patented technologies are 3D printed bioceramics [360] and porous titanium [361].

An alternative approach to ceramic scaffold-guided bone repair is the use of native collagen gels, reconstituted *in vitro* to encase viable cells for subsequent transplantation into a bone defect. Type I collagen is a natural polymer and the primary component of the organic network into which hydroxyapatite crystals, the mineral phase of bone, are deposited [362]. It has been widely investigated for its potential use as a scaffold for bone tissue engineering due to its high osteoconductive capacity and low immunogenicity. Osteoblast-like cells [363] or mesenchymal stromal cells (MSC) isolated from whole bone marrow [347] and seeded at high density in compressed collagen gels [364] differentiated over time down the osteogenic lineage [365]. The "plastic compression" approach thus yields a Type I collagen matrix with a fibrillar density similar to that of bone matrix and containing viable osteogenic cells. Although these cell-seeded dense collagen scaffolds have been shown to support mineralisation by osteoblastic cell lines and MSC *in vitro*, their capacity to repair bone in an appropriate pre-clinical model has not yet been demonstrated.

As is the case with bone development, bone regeneration is dependent on adequate and orderly recruitment of endothelial cells to form capillaries that will deliver oxygen, nutrients,

growth factors and precursor cells to the site of healing [118]. Vascular endothelial growth factor (VEGF) is a potent mediator of neo-vascularization of the growth plates in developing bones [366], as well as angiogenesis that occurs during fracture repair [367]. The aim of the current study was to examine healing of a 1 mm x 3 mm surgically induced window defect in the mid femoral diaphysis of skeletally mature C3H mice in response to MSC seeded dense collagen scaffolds. Integration of the scaffolds with native bone for optimal healing was promoted using a single, bolus dose of VEGF at a critical stage of callus formation.

MATERIALS and METHODS

Fig 5.1 outlines the experimental approach and work flow chart for the study.

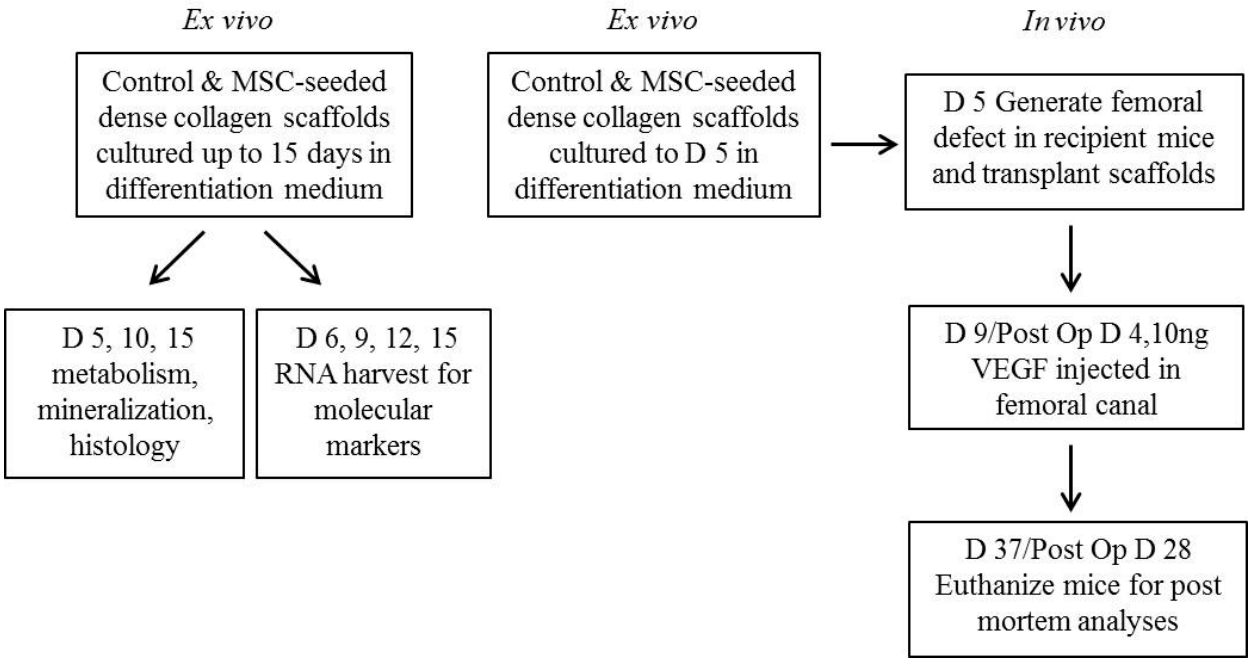

Figure 5.1 Experimental design and work-flow for *in vitro* and *in vivo* experiments:

Mesenchymal stromal cells (MSC) were harvested from skeletally mature donor mice and seeded in hydrated type I collagen gels. Unconfined compression of the gels yielded dense collagen scaffolds that were cultured and removed at timed intervals for *in vitro* evaluation of metabolic activity, mineralization, histological analysis or for RNA isolation and molecular studies. For *in vivo* studies, uni-cortical defects were drilled in the femoral diaphysis of recipient mice and filled with scaffolds pre-cultured for 5 days. Recipient mice were left for an additional 28 days for bone healing to take place.

Isolation of MSC and preparation of cell-seeded dense collagen scaffold

MSC were isolated essentially as described previously [368]. The soft tissue and the knee ends of the femora and tibiae were removed under aseptic conditions before placing the bones in Eppendorf tubes and centrifuging at low speed to dislodge the marrow. Bone marrow from individual 4 month old wild type C3H mice (total 3) was pooled, re-suspended in alpha MEM containing antibiotic/antimicotic (Sigma-Aldrich, St Louis MO) and 10% FBS (Wisent Inc., St Bruno, Quebec) and plated in 100 mm tissue culture dishes [71]. At 80% confluence each dish yielded ~ 6 x 10^6 adherent cells, which were trypsinized and used at first passage to prepare 21 cell seeded scaffolds (3 x 10^5 / scaffold) using a modification of a previously described method [365]. A solution of 2.2 mg/ml rat tail Type I collagen (First Link, West Midlands, UK) was diluted to 1.95 mg/ml with 10x DMEM (Sigma-Aldrich), and the pH adjusted to 7.5 with NaOH. Two hundred microliters of alpha MEM (Sigma-Aldrich) containing 3x10^5 MSC was added to each 800 microliters of pH adjusted collagen solution. The solution was then transferred into one well of a 24 well plate (diameter 16 mm) and incubated for 30 minutes at 37°C to complete gelation of the 16 mm x 5 mm (depth) hydrated gels. Control scaffolds were prepared with 200 microliters of alpha MEM alone. Collagen gels were subjected to unconfined compression under a load of 1.4 kPa for 3 minutes to expel water and generate the cellular or acellular (control) scaffolds measuring 16 mm x 0.5 mm (depth) with an estimated collagen fibrillar density of 4.8 wt %. Scaffolds were cultured in osteogenic alpha MEM supplemented with 10 % FBS, 50 microgram/mL ascorbic acid, 10 mM beta-glycerophosphate, with medium changes every 3 days for the duration of the experiment.

***In vitro* metabolic activity and differentiation of MSC in dense collagen scaffolds**

Metabolic activity was quantified on 3 gels (technical replicates) seeded with MSC from each of the 3 different mice (biological replicates) at days 5, 10 and 15 (total 9 gels / mouse) of

culture using the AlamarBlue® fluorescence assay according to the manufacturer's instructions (Life Technologies, Burlington, ON, Canada). After the metabolic assay the same scaffolds were fixed in 4 % paraformaldehyde for 30 minutes, washed in several changes of sterile PBS and stored in PBS at 4 °C for micro-CT imaging. Scaffolds were imaged with a Skyscan 1172 micro CT instrument (Skyscan, Kontich, Belgium) with no filter, a spatial resolution of 10 micrometers, a voltage of 50 KV and power of 10 W. Skyscan software was used for cross sectional reconstructions with NRecon, quantitative analysis with CTAn and 3D reconstruction with CTVol. Mineral content of the scaffolds was defined by setting the segmentation threshold between 120 and 255 in the binary mode of CTAn. The total volume of the scaffold and the volume of mineral in a defined region of interest (ROI) were quantified in 3 biological replicates at the indicated times. After micro CT analysis, the cell seeded scaffolds were embedded in paraffin and consecutive 5 micron sections stained with alkaline phosphatase (ALP) to assess osteoblast differentiation, Von Kossa/Toluidine blue to identify phosphate and Alizarin Red to identify calcium deposited in the collagen matrix.

For molecular analyses, RNA was extracted with Trizol® reagent (Invitrogen, Life Technologies Burlington, ON, Canada) used according to the manufacturer's instructions, on 3 technical replicates from each of the 3 different mice on days 6, 9, 12 and 15 (total 12 gels / mouse) of culture. Each scaffold was homogenized in 1000 microliters of Trizol using an RNase-free pestle and mortar on ice. 250 microliters of chloroform was then added and the mixture centrifuged at 11,000 rpm for 15 minutes. About 500 microliters of supernatant was transferred into clean RNase-free tubes and mixed with an equal volume of 70 % ethanol to precipitate the RNA. The RNA was then loaded onto an RNeasy Mini Kit (QIAGEN Inc. Toronto, ON, Canada) column and purified according to the manufacturer's instructions. The RNA extracted

from cells in a single gel was diluted in 35 microliters of RNase-free water for qPCR analysis. Reverse transcription was performed using a high capacity kit and expression of marker genes quantified using the Taqman assay (Applied Biosystems, Life Technologies, Burlington, ON, Canada). Expression of Type I collagen (Collagen I), Cbfa1, Osteocalcin, Osterix, PTH1R and MMP13 were normalised to that of the housekeeping gene glyceraldehydes 3-phosphate dehydrogenase (GAPDH) and presented as fold increase over day 6 values.

***In vivo* model to evaluate bone repair in response to cell seeded scaffolds**

Dense collagen scaffolds were prepared with or without MSC and cultured for 5 days in osteogenic medium prior to being trimmed, folded and implanted into freshly drilled femoral defects. All animal procedures were performed in strict accordance with a protocol approved by the McGill Facility Animal Care Committee, in keeping with the guidelines of the Canada Council on Animal Care. Rectangular window defects measuring 1 mm x 3 mm were drilled using a 1 mm bit (Maxtech Consumer Products Ltd, Waterloo, ON) in the lateral surface of the femoral diaphysis of 8-10 month old male or female C3H wild type mice. Where indicated, 10 microliters of sterile PBS containing $5x10^{-8}$ M recombinant mouse VEGF (R&D Systems, Minneapolis, MN) was introduced into the femoral canal at the level of the defect via a Hamilton syringe. Animals were randomised into one of the following groups: 1) no treatment (n=8); 2) dense collagen scaffold implant on day 0 (n=4); 3) cell seeded scaffold on day 0 (n=8); 4) VEGF injection on post-operative day 4 (n=10); 5) cell seeded scaffold on day 0 + 10 ng VEGF on day 4 (n-10). The mice were maintained post op with free access to food and water for 28 days, when they were euthanized and the femurs harvested, fixed in 4 % paraformaldehyde overnight, washed in several changes of PBS and stored in PBS at 4 °C for micro CT and histological analyses.

Quantitative micro CT analysis of bone repair in response to cell seeded scaffolds

Post mortem analyses were performed according to published protocols [71]. Intact femurs were first imaged using digital X-ray (Kubtec, Milford, CT US) before trimming and scanning with the Skyscan 1172 micro CT using an Al0.5 filter with a spatial resolution of 5.5 microns at a voltage of 50 KV and power of 10 W. The image dataset was analysed using NRecon, CTAn, and CTVol programs as described above for the mineralised scaffolds. In CTAn, a rectangular ROI measuring 2.9 mm x 0.9 mm x 0.77 mm was defined to cover the area of the window defect where the scaffold was placed and where repair took place. Quantification of bone mass and structural properties within the ROI was reported by CTAn in numeric format. These included percentage bone volume (BV/TV, propotion of defined volume of interest occupied bone), bone mineral density (BMD, volumetric density of calcium hydroxyappetite), trabecular number (Tb.N, the number of traversals across a trabecular structure made per unit length on a linear path through a trabecular bone region), trabecular separation (Tb.Sp, average thickness of space), trabecular thickness (Tb.Th, average local thickness of bone structure), trabecular pattern factor (Tb.Pf, index of bone connectivity), structural model index (SMI, indicator of relative prevalence of rod and plates in 3D structure) and degree of anisotropy (DA, measurement of 3D asymmetry or the presence or absence of preferential alignment of structures along a particular directional axis).

Histological analysis of bone repair in response to cell seeded scaffolds

After micro CT imaging the femurs were processed for histological analyses essentially as described previously [347]. Un-decalcified bones were embedded at low temperature in PMMA and serial 5 micron sections were stained with 5 % silver nitrate (Von Kossa) and counterstained with 0.2 % toluidine blue to distinguish mineral from soft tissue. Adjacent sections were stained

with Naphthol AS-TR phosphate (Sigma-Aldrich) in tris-maleate buffer pH 9.3 to identify alkaline phosphatase (ALP) activity in osteoblasts or with disodium Naphthol AS-TR phosphate, sodium nitrite and pararosaniline hydrochloride in acetate buffer pH 5.0 to identify tartrate resistant acid phosphatase (TRAP) activity in osteoclasts. A second group of bones was decalcified in 10 % EDTA and embedded in paraffin for immunohistochemical staining of CD34 (Goat anti rat antibody, 1:300 dilution, R&D Systems, Minneapolis, MN) positive vascular endothelial cells or osteocalcin (Goat anti mouse antibody, 1:400 dilution, Biomedical Technologies Inc., Stoughton MA) positive osteoblasts. Sections were counter-stained with methyl green.

Statistical analysis

In vitro assays (Fig 5.2 and 5.3, Table 5.1) were performed on 3 cell seeded scaffolds from 3 biological replicates at each time point. *In vivo* data (Fig 5.4) is representative of 4 animals in group 2 (empty scaffold) and 8 to 10 biological replicates in all other groups. SPSS (IBM) was used for global analysis of variance (ANOVA) with post hoc Tukey's "honest significant difference" (HSD) test to determine significance with a probability of $p < 0.05$.

RESULTS

In vitro characterization of MSC-seeded dense collagen scaffolds

In previous work, the hydraulic permeability of cell seeded dense collagen scaffolds was investigated and showed that increasing collagen density correlated with increased potential for the differentiation of MSC trapped in the fibrillar network [365]. In the present study, several modifications were made in the production of the dense collagen scaffolds (see Materials and Methods) to optimize the differentiation of the MSC and generate a scaffold with greater mechanical stability for *in vivo* manipulation. The reduction of AlamarBlue® reagent to a fluorescent red product by metabolically active cells embedded in the dense collagen scaffolds indicated no change in their viability for up to 15 days when cultured in osteogenic medium (Fig 5.2 graph). Sections of paraffin embedded gels stained for ALP (Fig 5.2a-c) showed little change in activity between day 5 (Fig 5.2a) and day 10 (Fig 5.2b) but then a significant decrease was seen by day 15 (Fig 5.2c). In contrast, adjacent sections stained with Von Kossa/toluidine blue showed a progressive increase in phosphate deposition (Fig 5.2d-f), which was accompanied by increased Alizarin Red staining of calcium (Fig 5.2g-1), in the absence of any apparent change in scaffold cellularity, as evidenced by hematoxylin and eosin stained histological sections (data not shown).

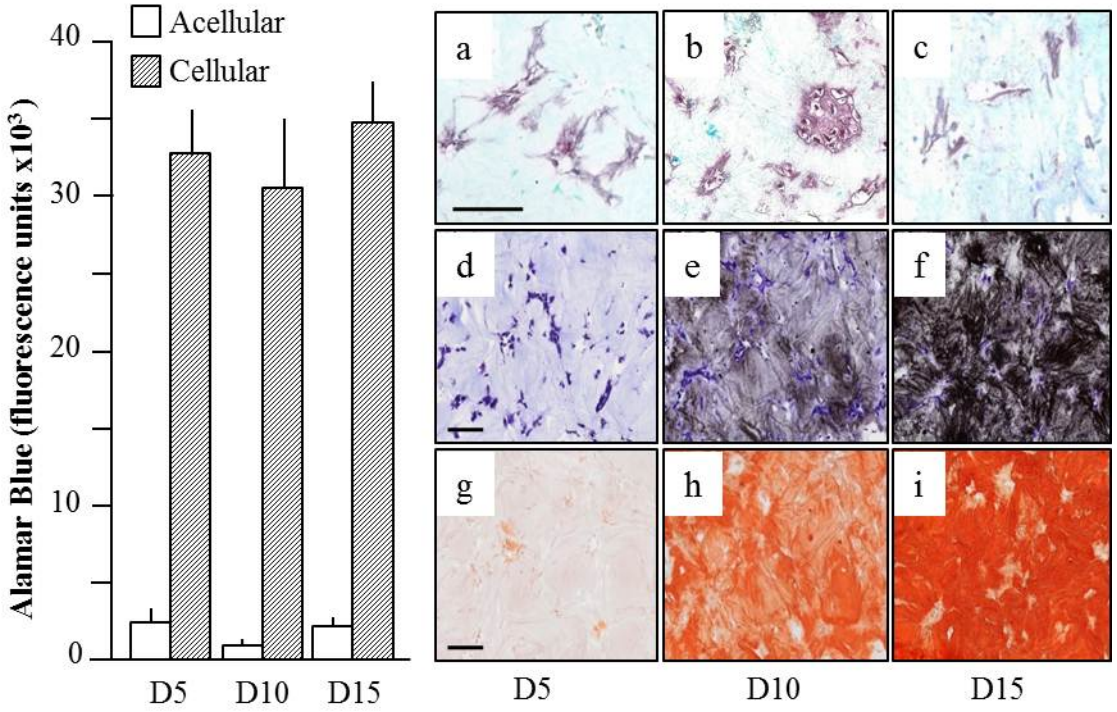

Figure 5.2 *In vitro* growth and differentiation of MSC in dense collagen scaffold: MSC were isolated from whole bone marrow of wild type mice by adherence to plastic and diluted at a concentration of 300,000/ml in 1.58 mg/ml Type I collagen solution. Hydrated collagen gels were subjected to a compression force of 1.4 KPa for 3 minutes and cultured in osteogenic medium. Alamar Blue was used to test the metabolic activity of cultures and 3 replicate gels removed at 5 days (a,d,g), 10 days (b,e,h) or 15 days (c,f,i) for histological analysis. Sections of paraffin embedded gels were stained with alkaline phosphatase (a-c) to monitor MSC differentiation, with von Kossa/Toluidine Blue (d-f) to identify phosphate or Alizarin red (g-i) to identify calcium deposited in the collagen scaffold. Scale bars represent 100 microns.

Quantitative PCR analysis (Table 5.1) of RNA extracted from the embedded cells after 6, 9, 12 or 15 days showed a time dependent up-regulation of markers of osteoblast differentiation, including type 1 collagen, the receptor for parathyroid hormone related protein (PTH1R), the matrix protein osteocalcin and the transcription factor osterix. Expression of the transcription factor Cbfa1 and matrix metalloproteinase 13 decreased over the same timeframe, but with a significant increase in Cbfa1 on day 15. Taken together with the increase in matrix phosphate and calcium, these results suggested a progressive increase in mineral deposition by osteogenic cells that had differentiated from the MSC entrapped in the collagen scaffold.

Table 5.1: qPCR analysis of gene expression in MSC-seeded dense collagen scaffolds cultured in osteogenic medium

Gene	Day 6	Day 9	Day 12	Day 15
Cbfa1	1.01 ± 0.16	0.73 ± 0.06[b]	0.62 ± 0.13[b]	1.20 ± 0.10[a]
Collagen I	1.01 ± 0.14	1.21 ± 0.23	2.86 ± 0.13[b]	2.06 ± 0.57[b]
MMP13	1.01 ± 0.12	0.57 ± 0.11[b]	0.23 ± 0.02[b]	0.33 ± 0.02[b]
PTH1R	1.00 ± 0.06	0.96 ± 0.11	2.27 ± 0.16[b]	2.02 ± 0.43[b]
Osteocalcin	1.02 ± 0.23	2.40 ± 0.77	9.49 ± 4.59[b]	13.30 ± 3.20[b]
Osterix	1.01 ± 0.17	1.19 ± 0.19	2.14 ± 0.50[b]	2.18 ± 0.41[b]

One-way ANOVA was used to analyze the differences in gene expression of cells embedded in dense collagen scaffolds harvested at 6, 9, 12 or 15 days of culture in osteogenic medium. Expression at day 9, 12 and 15 was compared with that at day 6 using Tukey's HSD test. Significantly different from day 6 [a] $p < 0.05$ [b] $p < 0.01$

A program written in the custom processing mode of CTAn software was used to delineate a region of interest (ROI) on the 2D reconstructed images of the scaffolds, shown in horizontal (Fig 5.3a) and vertical (Fig 5.3b,c) planes, for quantification of mineral based on differential X-ray attenuation in scaffolds cultured for 5, 10 or 15 days (Fig 5.3d-f). The quantitative data, shown in the graph, reveals an increase in the scaffold volume/thickness between day 5 and day 10 and no change thereafter. In contrast, the mineral content increased steadily over time to reach a more than 14 fold increase on day 15 over day 5.

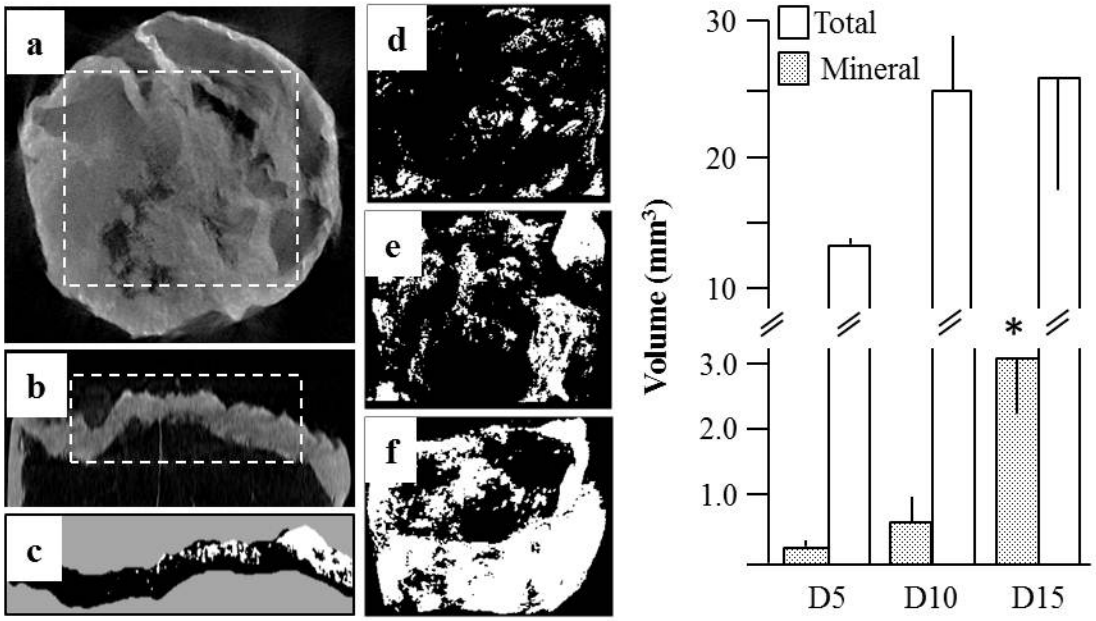

Figure 5.3 Quantitative micro CT analysis of scaffold mineralization: Dense collagen scaffolds seeded with MSC were prepared and cultured as in Fig 5.2 and 3 replicate scaffolds were harvested on day 5, 10 and 15 for micro CT analysis of mineral deposition. The 5 mm x 5 mm volume of interest (VOI) is shown on a 3D reconstruction of a scaffold in the horizontal (a) and vertical (b, c) planes. Mineral content is shown in white on representative 3D images, binarized in CTAn, in the vertical plane (c) and in the horizontal plane on Day 5 (d), day 10 (e) and day 15 (f). Quantitative data, expressed as the mean ±SD of three replicate scaffolds, for total scaffold volume compared with the mineralized volume is shown for the different time points. Significantly different from day 5 *$p < 0.05$

Bone repair in response to implantation of MSC-seeded dense collagen scaffold *in vivo*

Data from a previous study [365] and from the *in vitro* data from this study indicated that day 5 of culture marked decreased proliferation and the onset of differentiation of the MSC seeded in scaffolds. To optimize conditions for bone repair, the scaffolds were therefore cultured for 5 days before loosely rolling and implanting them in 1 mm x 3 mm drill-hole defects in the femoral mid-diaphysis of skeletally mature mice (Fig 5.4a-c). Given the critical role played by neovascularization in bone repair, some of the defects were treated with a single dose of VEGF in the presence or absence of cell-seeded scaffolds at post-operative day 4 (Fig 5.4d) and bone repair evaluated at post-operative day 28 (Fig 5.5).

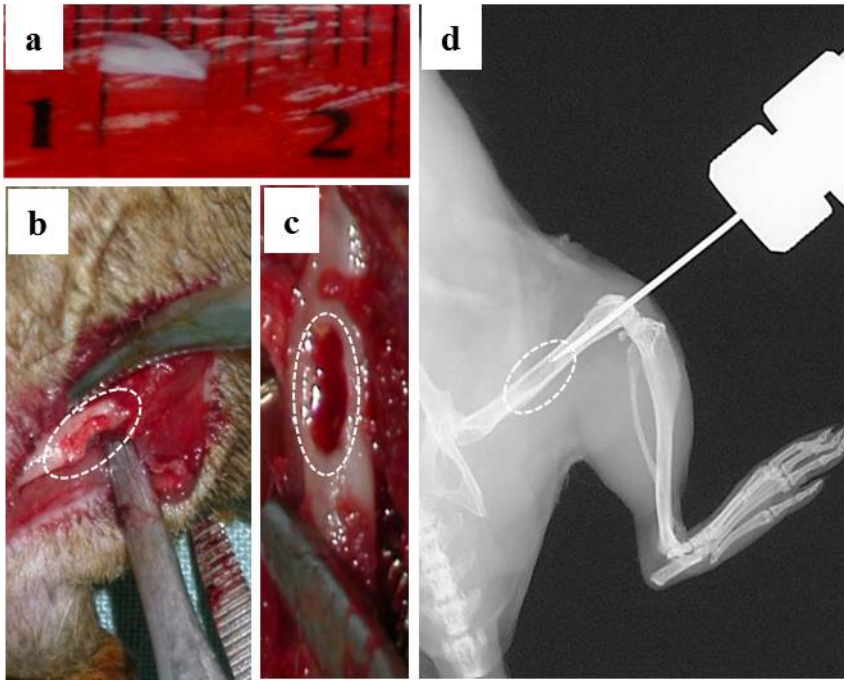

Figure 5.4 Femoral window defect and VEGF administration: MSC-seeded scaffolds prepared as described in Fig 5.2 and cultured *in vitro* for 5 days were loosely folded (a) before placing carefully in 1 mm x 3 mm window defects drilled into the mid-diaphysis of the femur in 8-10 month old wild type C3H mice (b-c). On post-operative day 4, 10 microliters of PBS containing 10 ng of recombinant mouse VEGF was injected into the intramedullary canal (d) via the inter-condylar notch using a Hamilton syringe. Femurs were harvested for analyses at post-operative day 28.

High resolution plain X-rays showed little difference between femurs receiving the different treatments (Fig 5.5a-e). High resolution 2D micro CT images (Fig 5.5f-j) showed little healing of defects with no treatment (Fig 5.5f), those treated with an empty dense collagen scaffold (Fig 5.5g) or with an MSC-seeded scaffold (Fig 5.5h). There was some healing in the presence of VEGF (Fig 5.5i) with significantly more in those defects treated with the combination of cell-seeded scaffold and VEGF (Fig 5.5j). The corresponding thin sections of plastic embedded tissue stained with Von Kossa/toluidine blue (Fig 5.5k-o) confirm significant accumulation of mineral only in the defects treated with VEGF in the absence (Fig 5.5n) or presence (Fig 5.5o) of MSC. The boxed region in Fig 5.5o is shown at higher magnification in Fig 5.5p. Adjacent sections stained for alkaline phosphatase (ALP, Fig 5.5q) or tartrate resistant acid phosphatase (TRAP, Fig 5.5r) show significant ALP activity in osteoblasts and numerous TRAP positive osteoclasts at the junction of mineralized and un-mineralised scaffold (arrows).

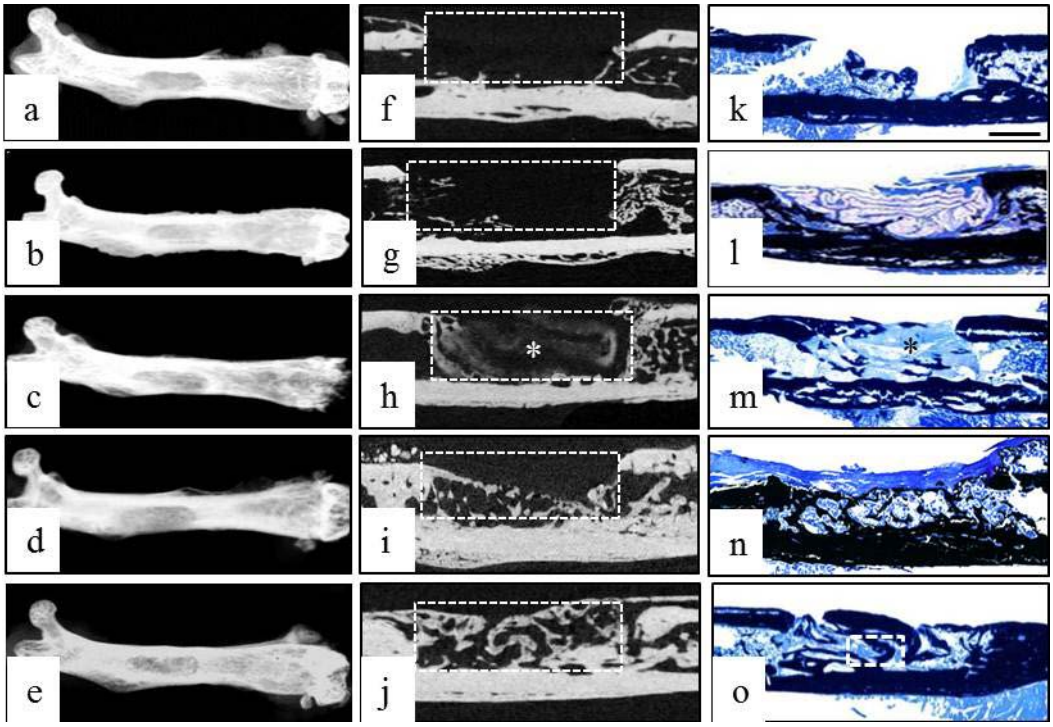

Figure 5.5 Radiologic and histologic analyses of bone repair: Representative X-ray (a-e), micro CT (f-j) and plastic embedded calcified bone stained with Von Kossa / Toluidine Blue (k-o) images are shown of bone repair at 28 days post-operative for defects receiving no treatment (n = 8 a,f,k), collagen scaffold alone (n = 4 b,g,l), MSC-seeded scaffold (n = 8 c,h,m), 10 ng VEGF alone (n = 10 d,I,n) or both MSC-seeded scaffold and VEGF (n = 10 e,j,o). The asterisk (h,m) indicates the rolled MSC-seeded scaffold and the white dotted line (f-j) delineates the 2.9 mm x 0.9 mm x 0.8 mm region of interest (ROI) for the quantitative micro CT analyses shown in Fig 5.6.

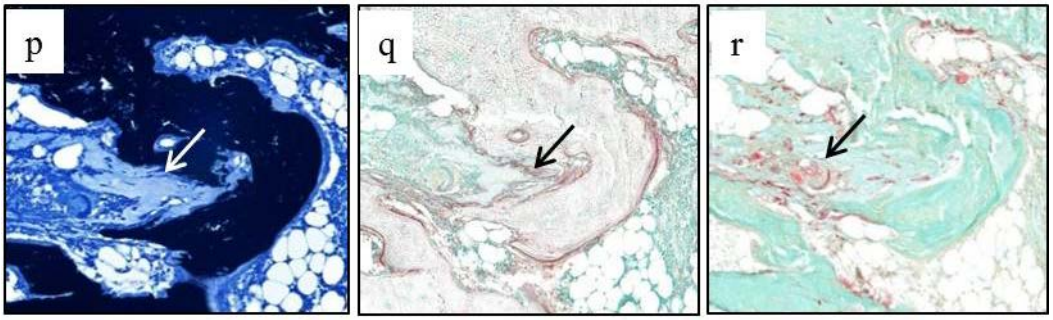

Figure 5.5 Radiologic and histologic analyses of bone repair (Cont'd): The area contained within the white dotted line in o is shown at higher magnification (p), along with sections stained for alkaline phosphatase (ALP, q) and tartrate resistant acid phosphatase (TRAP, r). ALP positive osteoblasts and TRAP positive osteoclasts were identified at the junction (arrows) of un-mineralized and mineralized scaffold. Scale bars represent 500 microns (k-o) and 50 microns (p-r).

Quantitative analysis of 3D micro CT data (Fig 5.6) revealed a significant improvement in bone volume relative to tissue volume (BV/TV), bone mineral density (BMD), trabecular number (Tb.N) and trabecular separation (Tb. Sp) in the group receiving the combination of cell-seeded scaffold and a single post-operative dose of VEGF (Fig 5.6e) compared with the control femurs receiving no treatment (Fig 5.6a). The differences in these parameters did not reach significance for the other treatment groups except for an increase in BMD in response to a single dose of VEGF. There were no significant differences (data not shown) in trabecular thickness (Tb.Th) or in indices of bone architecture including degree of anisotropy (DA), trabecular pattern factor (Tb.Pf) or structure model index (SMI).

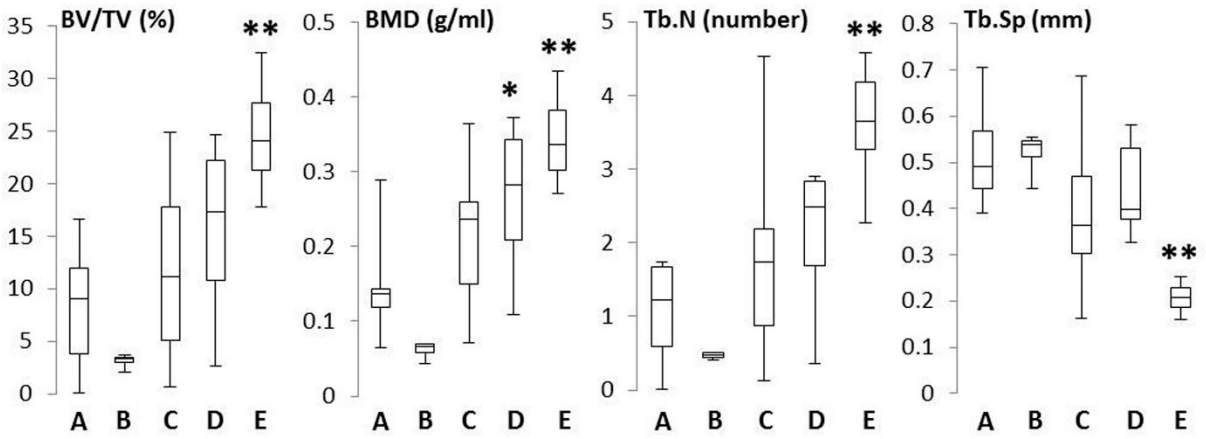

Figure 5.6 Quantitative micro CT analysis of bone repair: All femurs were scanned on a Skyscan 1172 instrument with a spatial resolution of 5.5 microns. Using CTAn analytical software, bone parameters were quantified in a rectangular region of interest (ROI) measuring 2.9 mm x 0.9 mm x 0.8 mm occupied by the scaffold. Values for bone as a percent of tissue volume (BV/TV), bone mineral density (BMD), trabecular number (Tb.N) and trabecular separation (Tb.Sp) are shown for femurs receiving no treatment (A), empty collagen scaffold (B), MSC-seeded scaffold (C), VEGF alone (D) or MSC-seeded scaffold and VEGF (E). Significantly different from A * $p<0.05$; ** $p<0.001$.

To further explore the increase in mineral content of the defects treated with cell-seeded scaffolds and a bolus dose of VEGF, representative specimens were decalcified and embedded in paraffin for immunohistochemical analyses (Fig 5.7). Sections stained for CD34 (Fig 5.7a-d), which is selective for hematopoietic progenitors and the endothelium of small vessels, revealed numerous positive cells within bone marrow adjacent to regenerating bone in animals that received no treatment (Fig 5.7a boxed area and a_1). CD34 positive cells were significantly increased in mice that received a bolus dose of VEGF alone (Fig 5.7c and c_1). There was a noticeable absence of CD34 positive cells in the specimens containing an empty scaffold (Fig 5.7b and b_1) but they were seen amongst the folds of the scaffold in animals treated with MSC-seeded scaffolds and VEGF (Fig 5.7d and d_1).

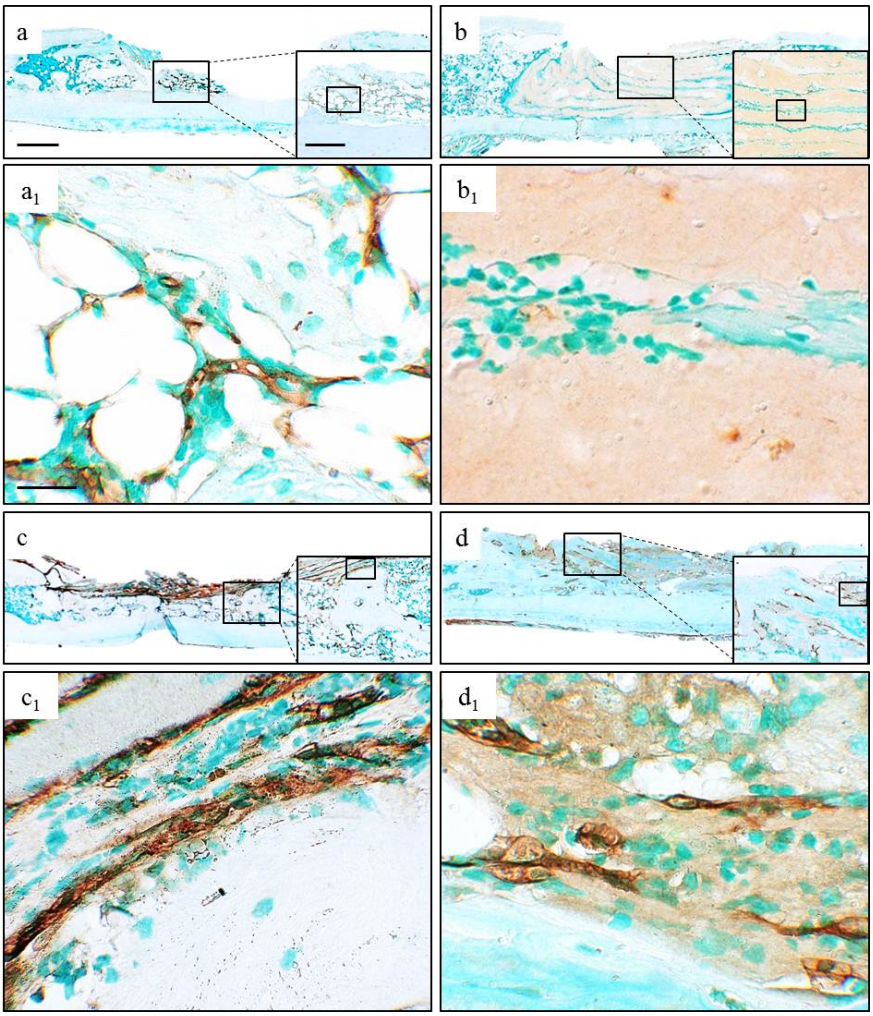

Figure 5.7 Immunohistochemical analysis of vascularity: Sections of decalcified, paraffin embedded bones were stained with CD34 antiserum (a-d) to identify vascular endothelial cells. Vascular endothelial cells (brown stain) were localized in regenerating bone on representative specimens with no treatement (a and a_1), those receiving VEGF alone (c and c_1) and those treated with MSC-seeded scaffold and VEGF (d and d_1) but not in specimens receiving collagen scaffold alone (b and b_1). Boxed areas in the insets indicate regions shown at higher magnification in the corresponding panels marked with "$_1$". Scale bars represent 500 microns (main panel-f); 200 microns (insets) and 20 microns (large images).

Osteocalcin is a non-collagenous protein that has been implicated in tissue mineralization and is deposited in bone matrix by mature osteoblasts. Minimal staining for osteocalcin was evident at the scaffold/bone interface in specimens containing an empty scaffold (Fig 5.8 a and a_1). However, osteocalcin staining was seen deposited between the folds of the cell-seeded scaffold (Fig 5.8b_1 asterisk) and at the junction of the mineralizing scaffold and native bone (Fig 5.8b_1 arrows).

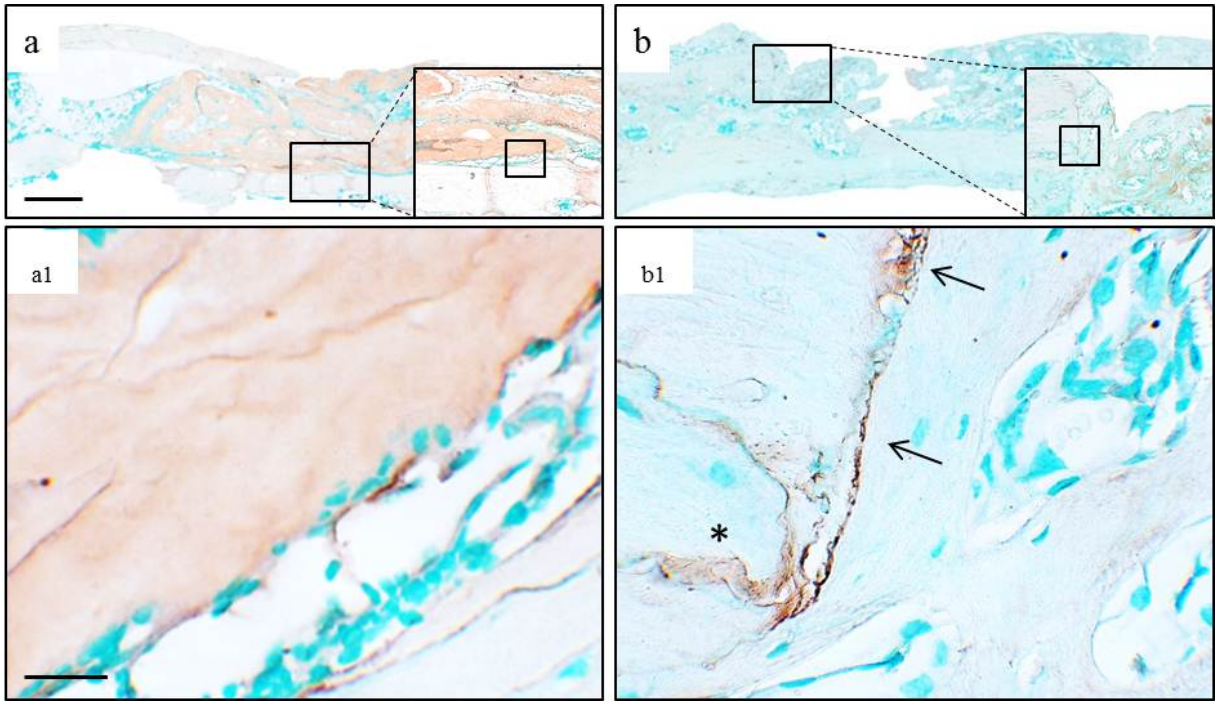

Figure 5.8 Immunohistochemical analysis of bone formation: Sections of decalcified, paraffin embedded bones were stained osteocalcin antiserum (a-b) to identify osteoblasts. A few osteocalcin-positive cells were seen at the periphery of the dense collagen scaffold (a_1) with more extensive deposits within the scaffold (asterisk) and at the bone-scaffold interface (arrows) in specimens treated with MSC-seeded scaffold and VEGF (b_1). Boxed areas in the insets indicate regions shown at higher magnification in the corresponding panels marked with "$_1$". Scale bars represent 500 microns (main panel-a); 200 microns (insets) and 20 microns (large images).

DISCUSSION

Bone repair in the adult skeleton begins with a prototypical inflammatory response to injury during which MSC are recruited from the sub-periosteum, the bone marrow and adjacent soft tissue to form a soft callus [59]. During the consolidation stage, MSC in this granulation tissue differentiate under the influence of local cytokines and growth factors into cells that form new trabecular and cortical bone. The number of cells and bioactive factors required for bone regeneration decline with age, in chronic disease or following treatment for inflammatory or neoplastic disease, which compromises the repair process and leads to bone necrosis and non-union of fractures [6]. An extensive literature has accumulated over several decades that documents attempts to promote bone regeneration using scaffolds, cells, cytokines and growth factors. A recurrent theme throughout this literature is the need for *in vivo* validation of novel therapeutic approaches in clinically relevant models.

Genetically modified and inbred strains of mice have been used with success for more than three decades to map the regulatory pathways involved in bone development and skeletal metabolism and many of these pathways are re-capitulated during bone regeneration in the adult. Despite their small size, this extensive body of knowledge has identified mice as a preferred species for *in vivo* analyses of bone repair [369]. A mouse model of a segmental defect with external fixation [370] has been used extensively to model non-union but confounding factors arising from high mobility made it unsuitable for study. We therefore modified a cortical drill-hole defect [371] to generate a reproducible, mechanically stable defect, where both cortical bone and endogenous bone marrow were disrupted. The higher bone mineral content, density and biomechanical strength of cortical bone in C3H mice compared with other mouse strains [372]

allows for generation of a 1 mm x 3 mm uni-cortical defect that is stabilized by the remaining cortex and will not heal without therapeutic intervention [373]. The study builds on previous work that characterised the *in vitro* differentiation of MSC in a dense collagen scaffold [365] by investigating the potential of cell-seeded scaffolds for bone healing *in vivo*. The results indicate that MSC seeded dense collagen scaffolds optimised for the osteogenic differentiation of MSC and then transplanted into femoral window defects will mineralize over time and integrate with native bone in the presence of VEGF.

An ideal scaffold for bone tissue engineering should be osteoinductive, osteoconductive and subject to resorption and replacement by functional site-specific bone over time [374]. Hydroxyapatite-tricalcium phosphate (HA-TCP), alone or in combination with autogenous bone, has been under investigation for more than three decades for this purpose [375] [376]. More recent studies have focused primarily on the use of calcium and magnesium based scaffolds and cements for the controlled release of bioactive molecules, including antibiotics and anti-neoplastic agents, into the bone micro-environment [377] [378] [162]. A major disadvantage of calcium phosphate based scaffolds is resistance to resorption by osteoclasts, unless they are pre-treated with an osteoclast activator such as RANK ligand [361], which has severely limited their clinical utility. Modification of their surface composition and topography with silk, which has a fibrillar structure similar to that of native collagen, has improved both the mechanical properties and biocompatibility of biphasic calcium phosphate scaffolds [378]. However, the increase in manufacturing complexity of composite scaffolds raises the cost considerably, which will ultimately be passed on to consumers in the healthcare industry.

To avoid the problems associated with traditional ceramic scaffolds and to develop a mechanism for introducing osteogenic cells into poor quality bone, a natural polymeric scaffold

that resembles the organic or "osteoid" phase of native bone was selected for the current studies. Due to their inherent fragility, hydrated type I collagen scaffolds are not commonly associated with hard tissue engineering applications but are commercially available for soft tissue engineering, most notably skin. In 2004 Professor R.A. Brown at University College, London developed a dense cellular scaffold with useful mechanical properties and ultrastructure simply by rapid expulsion of fluid from hydrated collagen gels using plastic compression [363]. In an *in vitro* study we recently demonstrated long term viability and osteogenic differentiation of MSC in dense Type I collagen scaffolds [365]. The ultra-rapid engineering and prior clinical approval for the use of collagen-based wound dressings make this an attractive option for bone tissue engineering. For the current application of cortical bone healing, pilot studies were conducted with variable compressive force and time, as well as different MSC seeding densities to generate a construct with optimal osteogenic properties. To enhance the mechanical properties of the scaffold while maintaining adequate diffusion of oxygen and nutrients, the compressive force and time were first increased by ~30 % while maintaining the MSC concentration at 6×10^6 /ml dense collagen. This led to minimal scaffold mineralization when implanted in the murine window defect and effectively formed a barrier to the influx of endogenous cells at postoperative 4 weeks. Based on previous *in vitro* work with osteogenic MG-63 osteosarcoma cells [364], a seeding density of 3×10^5 MSC/ ml of hydrated collagen was selected for a final concentration of 3×10^7/ ml dense collagen.

In our *in vitro* studies, this construct with a 5 fold increase in cell numbers and improved mechanical properties, supported the long term metabolic activity and differentiation of MSC into osteoblasts that deposited a calcium-phosphate rich mineral in the extracellular matrix. Increased expression of recognized molecular markers of osteoblast differentiation (type I

collagen, PTH1R, osteocalcin, osterix) were accompanied by reductions in Cbfa1 and MMP13 up to Day 12 with an increase on day 15 as reported previously [228 379]. Unfortunately, when implanted in the femoral window defects it acted as a barrier, separating the transplanted and endogenous cells. Insufficient vascularization for effective mass transport of oxygen, nutrients and waste products through the scaffold has been documented as an obstacle to integration of cell-seeded polymeric scaffolds into native bone *in vivo* [380]. The angiogenic properties of vascular endothelial growth factor (VEGF) are well recognised in both bone development [58] and bone repair [367 381]. It is a potent inducer of endothelial cell proliferation and migration and has been used in combination with a variety of biomaterials, including Type I collagen, to promote angiogenesis [382]. When rhVEGF was covalently linked to a commercial collagen sponge to achieve sustained release kinetics, it enhanced vascularization and the repair of myocardium *in vivo* [383]. We hypothesized that VEGF-induced angiogenesis would improve integration of the dense collagen scaffold with native bone in the healing window defect, in a manner similar to vascular invasion of the growth plate during long bone development [366]. A single, intra-medullary therapeutic dose of VEGF was therefore administered at postoperative day 4, which is the stage of fracture repair at which a hematoma has formed and an inflammatory response initiated. In the early phase of fracture repair a coagulation cascade is triggered and inflammatory cells migrate to the site where they release cytokines such as IL-1 and IL-6, and platelets also release important signalling molecules including PDGF and TGF-β. Although the mechanism of VEGF action under the present circumstances is not fully characterised, Fig 5.7 shows vascular endothelial cells and bone regeneration were increased at 28 days post-operative in the defects that received VEGF on day 4 post-operative. In the case of the defect that received both the cell-seeded scaffold and VEGF. it is possible that the inflammatory events in adjacent bone somehow

modified the dense collagen scaffold. Alteration of the scaffold might allow tethering of the injected VEGF, thus protecting it from degradation and enabling a more sustained release. Alternatively, the VEGF could have been tethered by structural molecules such as fibrin in the hematoma and released later to induce neo-vascularization [384] [385]. In fact, Fig 5.7 shows quite clearly that VEGF acted synergistically with the cell seeded dense collagen scaffold to induce bone formation.

The presence of mineralized scaffold in the centre of the defect and some remaining un-mineralized scaffold at the border with adjacent bone indicates that osteoinduction was initiated by cells trapped in the scaffold in an "inside out" manner. Of additional interest was the robust ALP staining within and at the periphery of the healing defect at 28 days *in vivo* whereas there was a decline in ALP activity *in vitro* at 15 days. Together with the presence of TRAP positive osteoclasts in and around the scaffold, this suggests active matrix turnover and release of signalling molecules that would promote osteogenic cell migration, vascular invasion and enhance mass transport. The osteoconductive properties of the mineralized scaffold, aided by VEGF, would thus enable migration and attachment of endogenous bone forming progenitor cells by way of the newly formed vessels. The presence of both osteoblasts and osteoclasts in central and peripheral areas, as well as osteocalcin positive "osteocyte-like" cells embedded in mineralised matrix, further suggests the extracellular matrix within the bone defect was undergoing robust turnover. Additional studies aimed at determining the fate of transplanted MSC and their relationship with endogenous cells during fracture repair will require the use of GFP or other labelled cells in the scaffold. A detailed time course, with bone healing examined as early as 4 days, and at weekly intervals thereafter up to 8-12 weeks, in the presence and

absence of VEGF therapy will be necessary to reveal the molecular mechanisms underlying the increase in bone formation at 4 weeks seen in the current study.

CONCLUSION

The results of our study indicate that a type I collagen scaffold with the consistency of un-mineralised bone induced the osteoblastic differentiation of MSC when folded into a large defect in the mouse femur. Deposition of mineral within the scaffold effectively filled the defect with “tissue” that resembled trabecular bone. A single post-operative dose of recombinant vascular endothelial growth factor promoted neo-vascularization and integration of the mineralised scaffold with endogenous bone that formed around the implant. The approach therefore represents a rapid, safe and cost effective mechanism for bone tissue engineering where endogenous mechanisms are compromised.

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Chapter VI: VEGF Covalently Tethered in Collagen Sponge to Expedite the Healing of Critical-Sized Mandible Defects

The work in the previous chapter indicated that the addition of an angiogenic factor, such as VEGF, is indispensable for expedited bone defect healing by means of *en bloc* bone formation. The most common approach for administrating VEGF is a direct local injection under the guidance of radiography, which is simple and replicable enough to be translated into clinical use; however, the dosage we used might be high when considering the animal weight and dimensions of the defect treated. The high dosage (0.3–0.4μg/kg) that we used can partially counteract the quick drop in the local concentration resulting from rapid diffusion, but some researchers have implied that this strategy may give rise to several complications. Traditionally, the controlled release of VEGF during the period of bone healing is preferred to the rapid diffusion associated with simple injection. This is particularly true for the repair of critical-sized bone defects as they require a longer period of healing time than non-critical-sized defects.

Inspired by the work of Dr. Ren-Ke Li, which involved rat myocardial repair by using VEGF covalently immobilized in a biodegradable collagen sponge, we explored the possibility of repairing critical-sized bone defects with the same collagen sponge that is tethered with VEGF and seeded with bone marrow-derived MSC.

I established the experimental protocols, conducted the bulk of the experiments, and performed data collection and analysis, as well drafting the paper. Dr. Janet Henderson provided essential supervision throughout the project and will edit my manuscripts for publishing. My peer graduate students, Zaher Jabbour and Saad AlQahtani, helped me in the

animal surgery. Brian P. Chen assisted with the histological assessment and manuscript preparation. Ailian Li and Huifen Wang provided technical assistance for the histological analysis and animal maintenance, respectively.

Covalently bonded VEGF promoted the repair of critical-sized mandible defect by MSC-seeded collagen sponge

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Running title: *Bone tissue engineering*

Keywords: critical-sized bone repair; collagen sponge; vascular endothelial growth factor, mesenchymal stromal cells

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ABSTRACT

Insufficient vascularization is a key pathogenic mechanism underlying nonunion of bone defect. It also imposes a major obstacle in engineered bone grafts designed to assist with bone healing. Vascular endothelial growth factor (VEGF) is a competent angiogenic factor that induces endothelial proliferation and functions synergistically with other osteogenic peptides to expedite ossification. The goal of this study was to investigate the potential of VEGF covalently tethered to a collagen sponge based engineered graft in promoting vascularization and bone regeneration of a critical-sized defect in the rat mandible. 4mm x 4mm critical-sized defects were drilled in the rat mandibles bilaterally. The RIGHT defect was treated with collagen sponge bound with VEGF and seeded with bone marrow derived mesenchymal stem cells (MSC). The LEFT defect was treated with collagen sponge without VEGF and seeded with the same bone marrow derived MSC. The collagen sponge with VEGF covalently tethered and implanted in the RIGHT mandible defect led to significantly greater bone formation at postoperative 8 weeks as determined by quantitative micro computed tomography (microCT) analysis. In addition, more vascularization around the defect at postoperative 3 weeks was shown by $BaSO_4$ perfusion assisted microCT imaging of vasculature and CD34 immunohistochemistry. VEGF covalently tethered to a collagen sponge scaffold showed promise as a novel biological cue to enhance vascularization of bone defect and expedite healing of fracture nonunion and critical-sized bone defect.

INTRODUCTION

The reconstruction of large bone defects is a common challenge shared by orthopedic, dental and oral-maxillofacial surgeons. A critical sized bone defect, by definition, will not heal spontaneously, and therefore requires intervention to promote bone regeneration [386] [387]. Bone grafting is a common procedure used to promote bone regeneration. Specifically, autograft is currently considered the gold standard for bone grafting as it satisfies all criteria of an ideal bone graft: osteoinductive, osteoconductive, biomechanically stable, disease free, and contains minimal antigenic factors [388]. However, supply is limited and the need for a separate operation at the site of tissue harvest leads to donor site morbidities [281]. An alternative approach is to use allografts, where tissue is obtained from a cadaver. While supply is more plentiful, its long term durability is questionable [389], and it carries a risk of disease transmission from donor to recipient [390]. Engineered bone graft substitutes represent a potential solution to circumvent the limitations of both autografts and allografts. Bone tissue engineering strategies have relied on introducing osteogenic cells and osteoinductive molecules to the site of a defect via an osteoconductive scaffold. Type I collagen has been used extensively to deliver bone morphogenetic protein2 (BMP2) [391], mesenchymal stem cells (MSC) [392], and other osteogenic or osteoinductive biologics to the site of the bone defect [20].

The positive effects of BMP delivered with a collagen sponge on bone healing is documented [393], and BMP-2 and BMP-7 delivered by absorbable collagen sponges was approved by the FDA for specific clinical uses in 2002 [394]. However, increased complications [288] [395] and serious side effects, such as increased risk of cancer [282], have been associated with its use. Clinical cases have not shown the same promise as pre-clinical models. Given these challenges,

there is a need to explore other osteogenic cues to promote bone repair. In this context, the application of MSC seeded in biomaterial scaffolds has been investigated to enhance repair of bone defects in the parietal bone [392] [396] and lumbar vertebra [397].

Numerous studies have also highlighted the role of vascular endothelial growth factor (VEGF) stimulated angiogenesis on bone healing [50]. We previously proved that the use of MSC-seeded type I collagen scaffolds along with a bolus dose of VEGF led to improved healing of a large non-critical sized window defect in mice femurs [398]. However, the VEGF treatment by direct injection necessitates extremely high dosages which are associated with increased cost and potential complications as mentioned above. Moreover, the injection of biologic solutions is followed by rapid diffusion with short duration of sufficient local concentration, resulting in failure to elicit the desired biological response. Indeed numerous authors have reported the methodologies of tethering VEGF covalently to collagen scaffold in simple lab settings that permit the prolonged in-vivo presentation of VEGF on the collagen carriers [383]. The reduced degradation and internalization promoted in-vivo repair of tissues such as infarcted myocardium. This approach of VEGF delivery appears promising in expediting bone healing, especially of critical-sized bone defects in which a longer healing time is required. The aim of the current study was to investigate the potential of VEGF covalently tethered to a MSC-seeded collagen sponge in expediting the healing of critical-sized defects in the rat mandible.

MATERIALS and METHODS

Generation of collagen sponge covalently tethered with recombinant VEGF

The Ultrafoam collagen sponge (Davol, Warwick, RI, USA) was cut into discs measuring 8mm in diameter with a biopsy punch and then subjected to VEGF cross linking by using 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) chemistry. The collagen sponge discs were first activated by immersing in 150µl of EDC/NHS (Sigma-Aldrich, St Louis, MO, USA) 24mg/60mg per ml of sterile PBS for 20 min at room temperature. Then they were soaked for 2 hours at room temperature in 100µl of 2µg ml^{-1} recombinant VEGF (R&D system, Minneapolis, MN, USA) dissolved in sterile PBS to form the covalent bonds with VEGF. Control collagen discs were soaked in sterile PBS without VEGF under the same condition. To remove the EDC, NHS and free VEGF, the sponge discs were washed in sterile PBS consecutively 8 times with a duration of 5 min for each wash. The VEGF cross-linked collagen sponge discs were transferred into warm alpha Minimal Essential Medium (αMEM) and then dabbed gently on sterile tissue to remove moisture and placed in 24-well tissue culture plates.

Seeding MSC into the VEGF-tethered collagen sponge

MSC isolation was done as described previously [368]. In brief, the femurs and tibias extracted from 4-month-old wild type C3H mice were cut into halves, placed in Eppendorf tubes with the osteotomy sides facing down and subjected to low speed centrifuge to dislodge the marrow. Total bone marrow cells were re-suspended in αMEM supplemented with 10 % fetal bovine serum (FBS) (Wisent, St. Bruno, QC, Canada) with antibiotic/ antimycotic activity (Sigma-Aldrich, St. Louis, MO, USA) and plated in 100 mm tissue culture dishes. The culture

medium was replaced every three days with mechanical dislodging of the non-adherent cells until adherent cells were 80 % confluence and ready for trypsinization and utilization at first passage.

The harvested bone marrow derived MSC were suspended in culture medium at a density of 20,000/µl. 30µl of MSC suspension was added to the top of each collagen sponge disc. After being incubated for 40 minutes to allow for the adherence of MSC, the MSC-seeded collagen sponge discs were cultured in the medium supplemented with 50 µg/mL ascorbic acid and 10 mM β-glycerophosphate for 5 days to induce osteoblast differentiation before in vivo study.

Establishment of pre-clinical rat mandibular model of critical sized bone defect

All animal procedures were performed in accordance to a protocol approved by the McGill Facility Animal Care Committee in keeping with the guidelines of the Canada Council on Animal Care. Sprague Dawley rats (4-5 months of age) were anesthetized by continuous inhalation of isoflurane, and the rat masseter was dissected along the muscle fibers until the mandible angle was exposed. The mandibular bone bed was cleared of attached soft tissue using sterilized gauze. A square defect measuring 4mm x 4mm was then drilled using a 1 mm bit (Maxtech Consumer Products, Waterloo, ON, Canada). A pilot group of rats (n=6) was used to ensure complete healing was not possible (ensuring critical size defect definition) and also to provide quantitative values of baseline bone regeneration of the critical sized defect at 8 weeks postoperatively. Following the pilot experiment, the same defects were drilled bilaterally into another cohort of rats (n=6). The LEFT side was treated with collagen sponge disc seeded with BM-MSC without VEGF and the RIGHT side was treated with collagen sponge disc seeded with bone marrow derived MSC and tethered with VEGF.

Assessment of in-vivo bone regeneration in the critical sized defects in the rats' mandibles

At postoperative 8 weeks, the 6 rats in the control cohort were euthanized by carbon dioxide inhalation, and both LEFT and RIGHT mandibles were extracted for post-mortem assessment. The mandibles were imaged using digital x-ray (Kubtec, Milford, CT US), fixed in 4% paraformaldehyde (4% PF) overnight, and washed in PBS twice before micro-CT scanning using Bruker-Skyscan 1172 (Bruker, Billerica, MA US). The acquired CT images were processed and analyzed using Bruker-Skyscan software. The scanning was acquired using an Al0.5 filter with a spatial resolution of 9μm at a voltage of 50 KV and power of 10 W. Images were reconstructed using NRecon followed by analysis with Dataviewer and CTAn. In CTAn, a square region of interest (ROI) measuring 4mm x 4mm was defined in each slide across the whole dataset to delineate the boundaries of the critical-sized defect, producing a volume of interest (VOI) measuring 4mm x 4mm x 1.15mm to measure bone mass (mm^3).

Evaluation of angiogenesis of bone defect mediated by VEGF covalently tethered with collagen sponge

The effects of VEGF on vascularization of the bone defect were investigated at postoperative 3 and 6 weeks. At each time point, 3 rats with critical sized defects drilled bilaterally in the mandible and receiving the aforementioned treatments were used for post-mortem vascular analysis. Mann–Whitney U test was used to compare the bone mass of treated and untreated defects while Wilcoxon Signed-Rank test was used for within animal comparison of bone regeneration in response to treatments with and without VEGF tethered collagen discs. Vascularization was assessed with microCT imaging assisted by contrast agent perfusion and immunohistochemical staining for CD34.

Vascular contrast agent was prepared as previously described [399 400]. In brief, 100ml of 5% gelatin (Sigma-Aldrich, St. Louis, MO, USA) dissolved in 0.9% normal saline solution /100 g $BaSO_4$ was manually ground using mortar and pestle for at least 15 minutes with addition of sodium citrate tribasic dehydrate (Sigma-Aldrich, St. Louis, MO, USA) 0.5 g/100g BaSO4 and D-sorbitol 1.5g/100g BaSO4 to reduce the solution viscosity. The contrast agent was then stirred at low speed on a hot plate at 37°C before it was used for intra-arterial perfusion.

The rats were euthanized by carbon dioxide 30 minutes after intra-peritoneal injection of heparin 5 U/kg to prevent blood coagulation. Immediately following euthanasia, 20G plastic catheters were inserted in the left and right common carotid arteries. The thoracic cages were open and apertures in the right heart were made to release the pressure in the circulation system. The catheters were sequentially flushed with PBS followed by 4% PF twice until there were no traces of blood coming out of the right heart apertures. Thereafter, vascular contrast agent prepared as above was perfused into both catheters. Perfusion was stopped when the contrast agent overflowed from the insertion site. Following perfusion, rat cadavers were placed on ice for 10 minutes to allow the gelatin containing $BaSO_4$ to harden inside the vasculature. Both sides of the mandibles surrounded by bulk of soft tissues were carefully excised, fixed with 4% PF overnight at 4°C and washed with PBS twice before microCT and histological analyses.

The acquisition parameters used for microCT imaging of vasculature were similar to the ones used for bone regeneration except for using an Al-Cu filter and 80KV voltage. This allowed less artefact reflection from the high x-ray attenuation associated with $BaSO_4$. Based on the difference in x-ray attenuation of $BaSO_4$ and bone, the vasculature around the mandibular bone defect was segmented and volume-rendering models were generated in CTVox software with the vasculature coded in red and the bone in white. The shadow of $BaSO_4$-enhanced vasculature can

be segmented from that of bone in CTAn software based on the difference in x-ray attenuation. CTAn software was used to quantify blood vessels within the volume of interest (VOI) measuring 4mm x 4mm x 1.15mm VOIs and centered at the bone defects.

After micro-CT scanning, mandible samples were decalcified in 10% EDTA for 4 weeks at 4°C and embedded in paraffin. Serial sections at 5µm thickness were obtained for immunohistochemical staining of CD34 with primary goat anti-rat antibody at the dilution of 1:300 (R&D Systems, Minneapolis, MN) to identify the vasculature. Adjacent sections were stained for the markers of osteoblast, alkaline phosphatase (ALP), and osteoclast, tartrate resistant acid phosphatase (TRAP), to allow comparative view of osteoblastogenesis and osteoclastogenesis associated with angiogenesis.

RESULTS

VEGF covalently tethered to MSC-seeded collagen sponge expedited bone regeneration of critical-sized rat mandible defect

At postoperative 8 weeks, high-resolution plain X-ray (Fig 6.1 A1 and A2), representative 2D micro-CT images (Fig 6.1 B1 and B2) and 3D microCT images (Fig 6.1 C1 and C2) showed smaller area defects remaining unhealed in the defects treated by MSC-seeded collagen sponge with VEGF covalently tethered (Fig 6.1 A2, B2 and C2) compared to the defects treated by MSC-seeded collagen sponge without VEGF (Fig 6.1 A1, B1 and C1). The white frames in C1 and C2 panels indicate the region of interest used for quantifying bone mass which was plotted as shown in the Fig 6.1. The MSC-seeded collagen sponge without VEGF led to a bone mass of 1.65±0.82 mm^3, which is comparable to that with no treatment (1.78±0.90 mm^3, p=0.931); however, MSC-seeded collagen sponge with covalently tethered VEGF resulted in a bone mass of 4.92±0.45 mm^3- significantly higher than no treatment (p=0.002) or the treatment without VEGF (p=0.043). Bone analysis at postoperative 8 weeks implied it was the VEGF tethered in collagen sponge that led to the expedited defect healing. Therefore, the mechanism by which the immobilized VEGF in the collagen sponge increased bone regeneration was explored in the earlier phases of the healing process by consecutive microCT vasculature imaging and histological assessment.

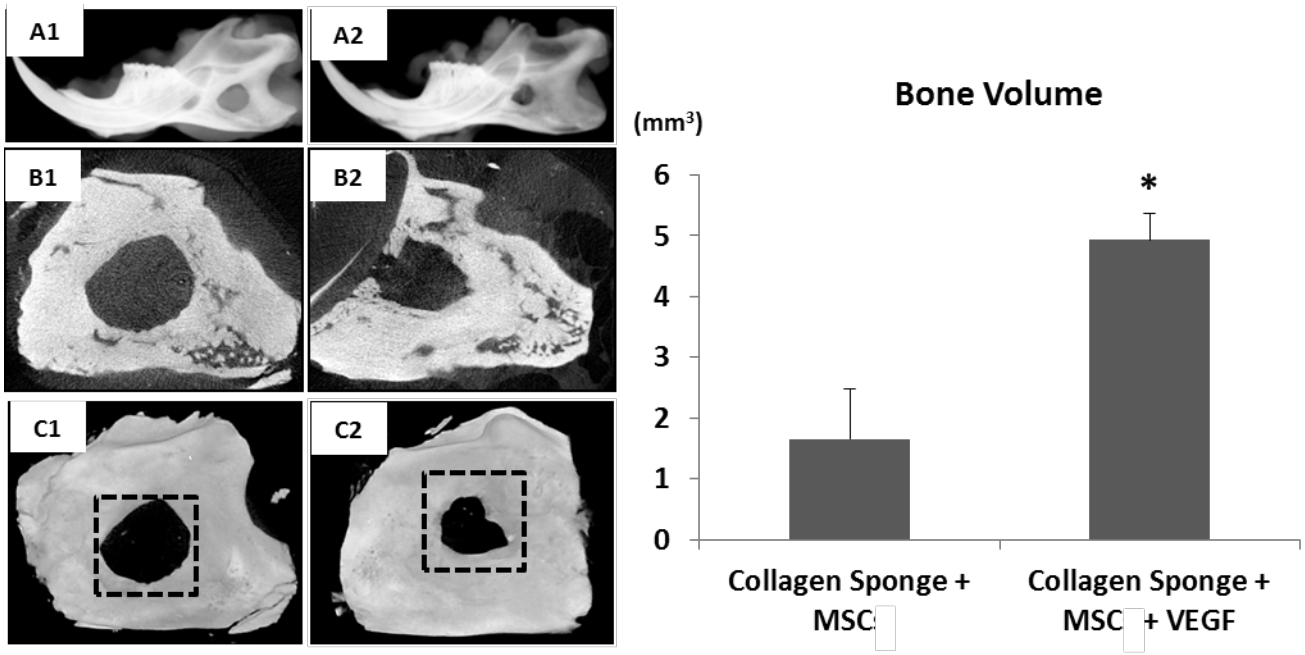

Figure 6.1 Quantitative microCT analysis of bone regeneration at postoperative 8 weeks: By plain X-ray (A1 and A2), 2D microCT images (B1 and B2) and 3D volume rendering images (C1 and C2), the healing of critical sized defects made in the mandible bones was compared among the groups receiving MSCs-seeded collagen sponge without VEGF covalently tethered (A1, B1 and C1) and MSCs-seeded collagen sponge with VEGF covalently tethered (A2, B2 and C2). The black frame drawn in C1 and C2 measured at 4mm x 4mm delineates the region of interest (ROI) used to quantify the bone mass which was plotted as shown. The VEGF-tethered MSC-seeded collagen sponge (C2) led to significant higher amount of bone mass than the one without VEGF tethered ($P<0.05$).

VEGF covalently tethered to collagen sponge increased angiogenesis

At both postoperative 3 weeks and 6 weeks, 3 rats were euthanized with the carotid arteries catheterized (Fig6. 2 A1) and perfused with BaS_4 solution dissolved in 5% gelatin (Fig 6.2 A2). The success of perfusion was monitored by real time intraoperative radiography that showed vasculature that was previously invisible prior to perfusion (Fig 6.2 B1) now enhanced by $BaSO_4$ (Fig 6.2 B2). Vascular quantification at postoperative 3 weeks showed remarkably higher vascular volume around the defects treated with VEGF compared to the defects treated without VEGF in 2 out of 3 animals (Table 6.1) while no visual difference in the healing of bone defects was observed (Fig 6.2 C1 and C2). At postoperative 6 weeks, the healing of the bone defects treated with covalently tethered VEGF appeared superior to the ones without VEGF treatment (Fig 6.2 D1 and D2) while no apparent difference was seen in the vasculature between them.

Table 6.1 MicroCT quantification of $BaSO_4$ enhanced vessel volume at postoperative 3 weeks and 6 weeks:

	3 weeks		6 weeks	
	Without VEGF	With VEGF	Without VEGF	With VEGF
1	0.03782	0.07486	0.10068	0.01665
2	0.16474	0.59697	0.14401	0.0568
3	0.10054	0.07809	0.10273	0.11422

Three rats (LEFT—MSC-seeded collagen sponge without VEGF and RIGHT—MSC-seeded collagen sponge with VEGF) were used at postoperative 3 weeks and 6 weeks for quantitative microCT analysis of vascularization with the aid of $BaSO_4$ perfusion. Quantification of vessel volume (mm^3) was performed within the VOIs that were centered at the defect and measured at 4mm x 4mm x 2mm. Due to the small sample size (n=3), VEGF covalently tethered to collagen sponge did not result in significantly higher volume of blood vessels at either 3 weeks and 6 weeks. However, the trend of higher volume of blood vessels formation by VEGF covalently tethered to collagen sponge was shown at 3 weeks.

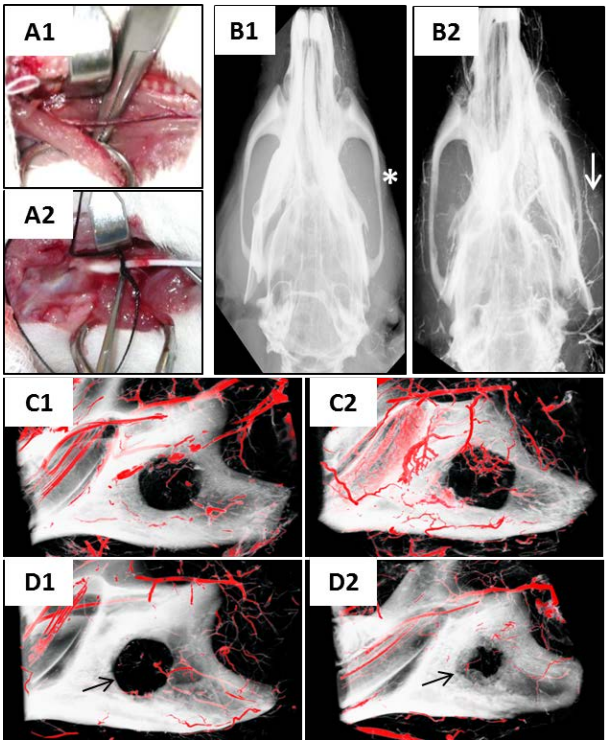

Figure 6.2 MicroCT analysis of vascularization mediated by VEGF tethered at postoperative 3 weeks and 6 weeks: After heparinization and euthanization, the carotid arteries were isolated (A1) and perfused sequentially by PBS, 4% paraformaldehyde and $BaSO_4$ dissolved in 5% gelatin (A2). Blood vessels were invisible in X-ray film prior to perfusion (asterisk in B1) and acquired enhanced X-ray attenuation after perfusion (arrow in B2). At postoperative 3 weeks, the MSC-seeded collagen sponge without tethered VEGF (C1) did not cause as much vascularization as MSC-seeded collagen sponge with VEGF (C2). At postoperative 6 weeks, no difference in vascularization was observed between the treatment of MSC-seeded collagen sponge without VEGF (D1) and MSC-seeded collagen sponge with VEGF (D2); however, the latter caused more advanced healing of the critical sized defect as indicated by increased newly formed bone structure within the defect (arrows in D1 and D2).

Further assessments of the vasculature following microCT imaging were done by immunohistochemistry staining of CD34. Adjacent sections were stained for ALP and TRAP to demonstrate the activity of osteoblasts and osteoclasts, respectively, in close proximity to vasculature.

At postoperative 3 weeks, newly formed bone, which has less dense architecture than the host bone, can be recognized at the edges of the defect (frames in the Fig 6.3 A1, A2, B1, B2, C1 and C2). These histological sections were imaged at higher magnification (Fig 6.3 a1, a2, b1, b2, c1 and c2) to document the neovascularization (Fig 6.3 a1 and a2) and activities of osteoblasts (Fig 6.3 b1 and b2) and osteoclasts (Fig 6.3 c1 and c2). More robust vessel formation was seen at the loci of osteogenesis in the defect that received MSC-seeded collagen sponge with VEGF covalently tethered compared to the one treated by MSC-seeded collagen sponge without VEGF (Fig 6.3 a1 and a2). In addition, weaker osteoblastic staining was noted at the edge of the bone defect that was treated without VEGF when compared to those treated with the addition of VEGF (Fig 6.3 b1 and b2). The stronger ALP staining lining the bone callus indicates that the larger number of osteoblasts at the of the defect peripheries (Fig 6.3 B2 and b2) is associated with enhanced vascularization mediated by the covalently tethered VEGF. Regardless of the types of treatments used, similar osteoclast activities were noted in the defects at postoperative 3 weeks (Fig 6.3 c1 and c2).

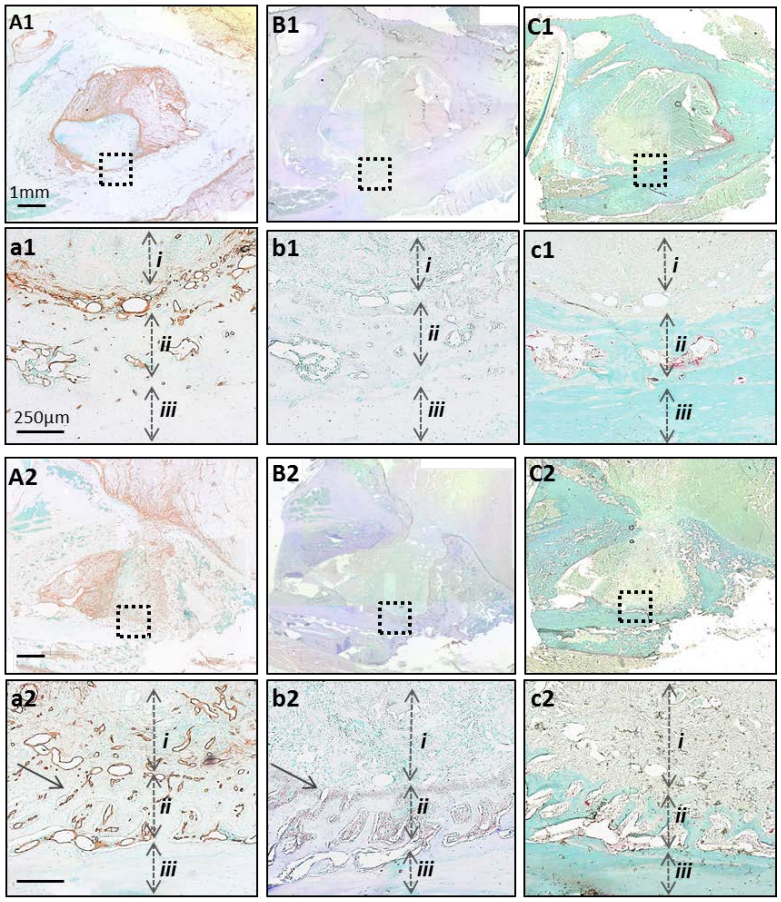

Figure 6.3 Histological analysis at postoperative 3 weeks: Decalcified mandible samples obtained from the treatment groups of MSC-seeded collagen sponge without VEGF (A1, B1, C1) and MSC-seeded collagen sponge with VEGF (A2, B2, C2) were analyzed by immunohistochemistry staining of CD34 to identify vessel structure (A1, A2), alkaline phosphatase (ALP) staining for osteoblasts (B1, B2) and tartrate resistant acid phosphatase (TRAP) staining for osteoclasts (C1, C2). The magnification images of the insets are shown (a1, a2, b1, b2, c1, c2). Magnified images illustrate the boundaries between defect (zone *i*), newly formed bone (zone *ii*) and host bone (zone *iii*). The covalently tethered VEGF in the collagen sponge led to more vessel formation adjacent to edges of the bone defect (arrow in a2) along with more osteoblastic activity (arrow in b2). However, no difference was observed in osteoclast activity.

The histological assessment at postoperative 6 weeks (Fig 6.4) revealed no difference in vascularization (Fig 6.4 A1, a1, A2, and a2) and osteoclast activities (Fig 6.4 C1, c1, C2, and c2) between the treatments with (Fig 6.4 A1, a1, B1, b1, C1, and c1) and without (Fig 6.4 A2, a2, B2, b2, C2, and c2) tethered VEGF. However, the tethered VEGF collagen sponge led to a higher degree of continuity of osteoblastic lining along the edge of the mandibular defects. These appeared more interrupted in defects that were treated by collagen sponge including MSC only (Fig 6.4 b1 and b2).

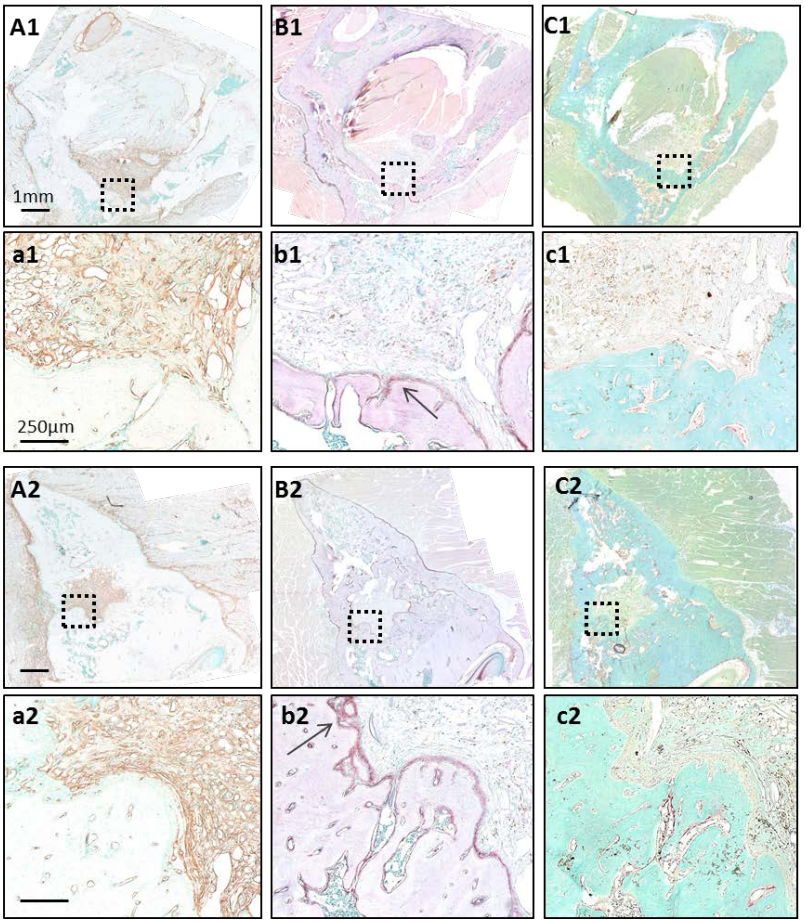

Figure 6.4 Histological analysis at postoperative 6 weeks: Decalcified mandible samples obtained from the treatment groups of MSC-seeded collagen sponge without VEGF (A1, B1, C1) and MSC-seeded collagen sponge with VEGF (A2, B2, C2) were analyzed by immunohistochemistry staining of CD34 to identify vessel structure (A1, A2), ALP staining for osteoblast (B1, B2) and TRAP staining for osteoclast (C1, C2). The magnifications of the insets are shown below (a1, a2, b1, b2, c1, c2). Magnified images show the boundaries between defect and newly formed bone. No obvious difference was observed in the vessel structure and osteoclast activity; however, continuous ALP positive osteoblastic lining along the edge of the bone defect was seen in the treatment including the VEGF arm with dispersed foci of ALP positive areas seen in the treatment excluding VEGF (arrows in b1 and b2 indicate osteoblasts).

DISCUSSION

In this study, we proved the therapeutic potential of covalently tethered VEGF in the healing of critical-sized defects in a rat mandible model as an extension of our previous work. We had previously illustrated that a single bolus dose of soluble VEGF can function synergistically with MSC-seeded collagen scaffold to expedite the healing of large but non-critical-sized defect in a murine femoral model [398]. Compared to non-critical-sized defects or simple fracture, critical-sized defects impose a larger biological challenge that impedes complete healing, requires longer retention of osteoinductive signals, a higher degree of vascularization, and a larger number of viable bone forming cells [401]. Several authors have implied the successful healing of acritical-sized bone defect by bone tissue engineering strategy necessitates sufficient induction of angiogenesis [117, 402, 403], and is believed to be the synergistic effects of a series of angiogenic factors including VEGF, angiopoietin-1, PDGF and bFGF [404]. VEGF signaling is recognized as the critical rate-limiting step in angiogenesis. VEGF165, the predominant isoform, can bind to Flt-1 (VEGFR-1) and Flk-1/KDR (VEGFR-2); aside from its interaction with NRP1 and NRP2 results in enhancement of VEGFR-2 signaling inside endothelial cells (EC). As a result, EC are mobilized to migrate and induced to divide which leads to the branching of existent vasculature and growth of new vessels [405]. Although the primary target of VEGF is EC, the roles VEGF played involve direct effect on non-endothelial cell types including osteoprogenitor cells [50]. It has been observed in several studies, including our previous study [398], that VEGF promoted mineralization in increase bone mineralization.

For almost a half century, BMPs have been the biologics most extensively studied for bone reconstruction procedures in both preclinical experiments and clinical trials. Together with high potency of BMPs shown in the preclinical models of small animal species are the convincing outcomes obtained from clinical trials, which led to the approval of BMP usage in several bone related conditions from several regulatory agencies, including US Food and Drug Administration (FDA) and European Medicines Agency (EMEA) [286]. The FDA approved rhBMP7 as an alternative to autograft in long bone nonunion and spinal posterolateral fusion as well as rhBMP2 for anterior lumbar interbody fusion (ALIFs) with a combined usage of titanium interbody cage, the spinal posterolateral fusion and open tibia fracture fixed with intramedullary nail [283]. In these clinical scenarios, supraphysiological dose of BMPs is necessary for eliciting desired biological response at the risk of inducing bone marrow fibrosis and sarcoma formation as well as at higher costs than that of autograft usage [288] [282, 395] [406]. Furthermore, limited success was achieved when using BMPs to repair large-sized bone defects in both animal models and clinical trials [289] [359] [407].

The carrier we chose for VEGF delivery was a collagen sponge that can also serve as supportive matrices for migrating cells. The porous collagen sponge is degraded in vivo at a rate that is tunable by adjusting the degree of crosslinking of collagenous fibers using chemical [408] [409] [410] and physical treatments [411] [412]. Initially being approved for hemostasis and wound dressing, the collagen sponge has also been used as carrier for rhBMP2 [285]. In fact, commercial rhBMP7 preparations also use collagen based carriers [413]. Prior to administration, the rhBMP2 solution is absorbed into collagen sponge which results in incorporation of rhBMP2 into the collagen sponge by its dissolution in the contained liquid and physical absorption onto the collagen sponge by electrostatic attraction forces [286]. This incorporation allows controlled release that

increases the local retention of rhBMP2 with improved biological effects. Similarly, VEGF that is immobilized onto collagen matrices physically as reported is released into surrounding tissues in a diffusion-controlled manner with a resultant increased angiogenesis [145 321 414]. In the case of repairing critical-sized bone defects, it is preferred that VEGF signaling is prolonged, and angiogenesis occurs inside the biomaterials used to fill the defect rather than the surrounding tissues. In a study using a myocardial infarction model, a collagen sponge disc measuring 8mm in diameter covalently immobilized 97.2±8 ng VEGF, which was measured indirectly by subtracting the VEGF in washing solution, quantified by ELISA, from the starting amount added to the reaction system as described above [383]. The immobilized VEGF on collagen sponge increased the vessel density of the collagen sponge carrier at both postoperative day 7 and day 28, which accounted for the final improved tissue repair [383]. As such, we hypothesized that the tethered VEGF on collagen sponge enables prolonged retention of VEGF in the lesion and guided vascularization into the defect area, and as a consequence, promotes the healing of critical-sized bone defects.

Another merit of this collagen sponge, aside from the ease by which VEGF can be covalently tethered, is the plausibility of seeding cells into the porous construct. In our study, bone marrow derived MSC from C3H mice was loaded on top of each collagen disc in suspension and, after waiting 30 minutes for cell attachment, cultured in osteogenic differentiation medium for 5 days before in vivo transplantation. However, the collagen sponge seeded with MSC failed to improve the healing of the mandible defect with comparable degree of repair to that receiving no treatment at all. Indeed this result is consistent with that obtained in a previous study using MSC seeded dense collagen scaffold to promote the healing of large defect in murine femurs. MSC transplantation alone is insufficient to repair bone defects of large

size in the absence of angiogenic cues. Nevertheless, other possibilities need to be excluded by further investigation in order to bear convincing explanations. First of all, we lack data to evaluate the ability of the collagen sponge to support osteoblastic differentiation of MSC seeded within it, although the evidence showing its capacity of accommodating the migrating endogenous cells and serving as carriers for biological molecules is available [383] [414]. Thus the viability and osteoblastic differentiation of MSC seeded in the collage sponge with or without tethering of VEGF should be evaluated *in vitro* over a time course. Another concern raised is the fate of transplanted xenogeneic MSC after its exposure to the recipient's immune system even though the immune modulatory capacity of MSC that leads to the suppression of lymphocyte proliferation *in vitro* and successful engraftment in the mismatched immunocompetent recipients *in vivo* has been well documented [415, 416]. Some authors suggested the existence of species-specific differences that may prevent cross-species engraftment [237] and even cause alloreactivity after transplantation between different strains of mice [235]. Also of note is that the 5 days of culture of MSC-seeded collagen sponge in differentiation medium *in vitro* before its transplantation *in vivo* may compromise the immune privilege of MSC as they are more committed to osteoblastic lineage [417].

While the VEGF tethered collagen sponge led to more vascularization at postoperative 3 weeks, no obvious difference in vascularization was observed at postoperative 6 weeks between the treatments with and without immobilized VEGF. According to the report by a previous author, the VEGF tethered on collagen sponge lost its bioactivity after 4 weeks of storage in PBS as demonstrated by less migration and proliferation of endothelial cells in the "aged" collagen sponge than that of the freshly prepared VEGF-tethered collagen sponge [383]. This inactivation of tethered VEGF might account for the lack of difference at postoperative 6 weeks. The rate of

collagen sponge degradation also influences the duration of VEGF presentation at the lesion. It has been revealed that the degradation of collagen sponge was minimal during the first 6 days of incubation, but more significant after 12 days [383]. Thus angiogenesis was initially induced by the immobilized VEGF, but as the collagen sponge degraded, VEGF was probably released, minimizing the extent of angiogenesis at postoperative 6 weeks. In our histology images at postoperative 3 weeks, we noticed the absence of collagen sponge in the defect, which led us to reach the conclusion that the collagen sponge tether with VEGF had been completely degraded by postoperative 3 weeks and the difference in the treatments was eliminated thereafter. The degradation properties of the collagen sponge should be evaluated by histological methods at the earlier time points and can be modified accordingly to tailor for specific needs by adjusting the crosslinking using chemical and physical processing.

The 4mm x 4mm bone defect made in the rat mandible is a reliable model of critical-sized defect that fails to heal completely without intervention. Although a smaller round defect was previously used to test iliac graft in rats [418], the square shape of defect used in the current study is easier to delineate using microCT software. The surgery of mandible defect in the current experiment offered the advantage of being less invasive for the animals; as a result, the bilateral mandibular defect was better tolerated than bilateral long bone defects and resulted in low mortality rate among the experimental rats.

In this study, we used $BaSO_4$ perfusion to perform post-mortem microCT imaging of vasculature as reported by previous authors. However, complete vascular perfusion can hardly be achieved given the dramatic increase in the resistance as the viscous contrast agent advanced into small vessels and the possible premature coagulation of the contrast agent within the vasculature. When it comes to microCT quantification of vasculature signal, the major limitation is that we

are currently unaware of the extent of the angiogenic field that is relevant for bone regeneration. As such, the definition of VOI for vascular quantification is more susceptible to subjective preference, which leads to major variance in the quantification result. Other issues of this approach of quantitative vasculature analysis using microCT include overlapping greyscale of bone and small vessels due to similar X-rays attenuation [399] and lack of the data reflecting the vascular functionality. These technical difficulties, together with the small number of animals in cohort, might have led to non-significant differences in vascular quantification; however, vasculature adjacent to the mandible defect was more prominent when the treatment including tethered VEGF at postoperative 3 weeks.

In summary, the collagen sponge seeded with xenogeneic MSC is incapable of repairing the critical-sized defect for some reason that should be further explored. By increasing angiogenesis at the edges of the bone defect at an early stage of healing, the addition of covalently tethered VEGF to the MSC-seeded collagen sponge expedited the healing of the critical-sized bone defect as proved by the measurement of bone volume within the defect at postoperative 8 weeks.

CONCLUSION

Collagen sponge with covalently tethered VEGF and seeded MSC promoted vascularization and bone regeneration at the edges of critical-sized bone defect in rat mandible. The ease of engineering the covalent bond between the collagen sponge and VEGF makes VEGF a potential therapeutic molecule applicable for the repair of large bone defects in patients. Further investigation of possible synergistic effect of VEGF combined with other osteogenic cues, including BMP and osteogenic progenitor cells, are warranted.

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Chapter VII: Discussion

My PhD work is a proof-of-concept study to assess the transplantation of MSC derived from donors' bone marrow for promoting bone regeneration and reconstruction. Current bone reconstruction procedures in orthopedic and dental surgeries frequently necessitate the use of both non-degradable metal prosthesis and/or degradable synthetic bone grafts to repair the bone defects that have various pathological properties. Significant advancements have been made in biomaterial engineering technologies and improvements in the bioactivity of both temporary and permanent implants, which have resulted in enhanced bone regeneration in the close proximity of the implants. The augmented bone formation induced by these engineered implants accounts for the improved osseointegration of metal prosthesis with a superior clinical outcome and timely bony substitution of biodegradable synthetic graft materials. However, the cell-based tissue engineering strategies to foster osteogenesis and osseointegration are largely in the preclinical and small-scaled clinical trial phases.

7.1. Summary and interpretation of significant findings

The MSC derived from bone marrow have shown promise as an ideal cell resource for bone engineering, which is attributed to their multi-lineage differentiation, immune-modulation, and sufficient availability; however, convincing data is lacking as to the efficacy of MSC-based bone tissue engineering strategies in relevant preclinical models of bone reconstruction. Thus, several animal models were developed to simulate the skeletal pathological conditions that require reconstructive operations to achieve healing (Appendix I). For my thesis work, I selected three rodent models as useful tools to investigate the bone engineering in osseointegration and bone healing. A mouse model of a femoral implant osseointegration was developed as a model

for human hip prostheses (Chapter III) and a second murine model was used to investigate the reconstruction of large-scale defects, such as those remaining after tumor resection or arising from major traumatic injuries (Chapter IV). A similar approach was used to investigate vascularization and bone healing in a rat mandibular defect as a model of reconstruction for critical-sized bone defects in the maxillofacial area (Chapter V).

The MSC were isolated from the total bone marrow cells of 4-month-old mice on C3H background by adherence to plastic and propagated *in vitro* at P0 before *in vitro* and *in vivo* studies. *In vitro* culture induced the expression of osteoblastic markers and the deposition of minerals by the MSC that were seeded on the implant grade titanium or in the dense collagen scaffold. To improve the osseointegration of the intramedullary titanium implant, MSC were locally delivered into the femoral canal by directly injecting the cell suspension before placing the intra-femoral implants. The femoral canal forms a natural closed space so that the MSC suspension is confined within the area where the bone regeneration is required. This approach for MSC transplantation avoids the complicated preparation protocols that will later result in decreased chances of contamination and the lowered cost associated with its application. In contrast, the large bone defect model made in the murine femur is an open space with contracting muscles adjacent to it, so the MSC need to be delivered through a solid scaffold to guarantee their sustained presence in the skeletal defect. Previously, our lab showed that the dense collagen scaffold is superior to the hydrated collagen gel in its support of the osteoblastic differentiation of MSC and in its great potential for bone tissue engineering while serving as a vehicle for MSC delivery and the repair of bone defects. Also, the ultra-rapid engineering process that suffices the homogenous seeding of MSC into the dense collagen scaffold with physiological collagen fibril

density circumvents the lengthy and complicated manufacturing process, and thus possesses a great potential to be translated into real clinical applications.

The animal model used to study MSC transplantation on a titanium coated intramedullary implant is characterized by osteopenia and impaired bone mineralization due to the homologous deletion of genes encoding FGFR3 on a C3H background. In addition, the recipient mice were subjected to sublethal irradiation on the hind limbs to ablate endogenous cells from residing in the bone marrow. As such, this model simulates the challenging task of osteointegration and the biological fixation of femoral prosthesis in patients, which is characterized by poor bone quality and an impaired bone regeneration capacity as seen in the elderly and those undergoing chemotherapy or radiotherapy. This model also removes the confounding factor, the endogenous bone healing mechanism, to lend better evidence that the allogeneic MSC injected into the femoral canal enhanced the formation of bone in close contact with the intramedullary implant. Therefore, MSC transplantation by local delivery is a plausible and promising solution to improve osteointegration and the biological fixation of the titanium prosthesis in patients whose bone healing capacities are damaged.

My colleague reported the formation of a thick layer of fibrosis surrounding the titanium coated implant frequently seen in the FGFR3-/- mice [307], which was probably due to an anomalous expression of integrin and a cell-substrate interaction derived from the absence of FGFR3 signaling [419 355]. As an end product of a chronic inflammatory response to a foreign body, fibrosis not only impedes the osseointegration of a prosthesis but also forms a major barrier to the performance of other medical apparatus, such as implantable biosensors and drug delivery devices [420]. Interestingly, in my study, no fibrotic reaction to an intramedullary implant was observed in the femurs receiving either donor MSC or a vehicle. This lack of fibrotic

reaction could be due to the sufficient conditioning by the targeted irradiation to suppress inflammatory reactions following surgical reaming and implanting. In addition, the immunomodulation of the nonspecific inflammatory reaction exerted by MSC could guide the immune reaction that is initiated by an injury from fibrotic tissue formation to bone regeneration and abrogate the peri-implant fibrosis as a consequence [421] [422]. The absence of fibrosis in the control femur receiving the vehicle alone could have resulted from the modulation of the nonspecific inflammation exerted by the transplanted MSC that migrated from the other femur, as suggested by non-human primate models [356]. The specific immune modulation exhibited by MSC has led to extensive clinical trials focusing on the systemic administration of MSC as a biological treatment for multiple autoimmune diseases. This finding in my research using this osseointegration model advocates an exploration of the local delivery of MSC in more clinical scenarios other than osseointegration; for example, it could be a viable approach for suppressing a fibrotic foreign-body reaction so to preserve the bioactivity of implanted medical devices.

Even though MSC transplantation enhanced bone formation around the intramedullary implant and improved osteointegration, when delivered to the large bone defect via the dense collagen scaffold, MSC contributed little to defect repair, although they exhibited robust osteoblastic differentiation in the same collagen scaffold *in vitro*. The inefficient repair by the MSC-seeded dense collagen scaffold alone was attributed to its suboptimal osteogenic ability *in vivo* and poor integration with the recipient bone. The addition of a single bolus of VEGF was sufficient to enhance the angiogenesis and osteogenesis of the MSC-seeded dense collagen scaffold and improve its integration with the bone callus formed by the endogenous cells. Interestingly, the VEGF treatment alone also failed to lead to significantly higher amounts of bone formation in this cohort, as did the MSC-seeded dense collagen scaffold alone, although it

did significantly increase the bone mineral density (BMD). Therefore, the synergistic combination of MSC seeded in a dense collagen scaffold and VEGF injected locally improved the bone healing process. Given sufficient vascularization, the transplantation of allogeneic MSC seeded in a dense collagen scaffold has shown promise as a bone tissue engineering strategy to repair large-scaled bone defects.

Paradoxically, MSC transplantation alone is capable of improving the osseointegration of the intramedullary implant; however, it cannot repair large bone defects unless angiogenic cues are added. This discrepancy can be explained by the different biological challenges associated with the two different models. With respect to osseointegration, the orthopedic or dental implant invariably has an initial seamless contact with the host bone tissue, and the region of bone regeneration is restricted at the interface between the bone and implant. The deposition of a layer of bone matrix on the surface of the implant, the cornerstone event that dictates the success of osseointegration, is a less challenging task for the transplanted MSC as opposed to the repair of a large bone defect for which a much larger amount of bone callus is needed to re-establish anatomical integrity. The increased volume of bone callus inevitably demands a higher degree of vascularization to preserve the viability of cells and complete the structural remodeling. Therefore, in my study, the essential role of the supplemental administration of VEGF to the engineered bone graft was more pronounced in the healing of large bone defects for which bulk bone tissue formation is required. Another outstanding difference in the application of MSC in these two models is the vehicles that carried the donor MSC. While the collagen solution satisfied the requirements for transplanting MSC into the femoral canal, the dense collagen scaffold was used to deliver and retain the MSC in the bone defect. The slow degradation of the dense collagen scaffold made it form a barrier between the endogenous cells and the transplanted

MSC, which impeded robust bone formation; therefore, an expedited degradation of the collagen scaffold was probably another mechanism by which the single dose of VEGF improved bone regeneration. In my study, the active osteoclasts stained positive for tartrate-resistant acid phosphatase (TRAP) were found to surround the transplanted dense collagen scaffold, which indicated an active turnover in the presence of the locally delivered VEGF. The reciprocal relationship between angiogenesis and osteoclastogenesis has been revealed by previous researchers [423] [424]; in addition, VEGF can enhance the survival and resorptive activities of osteoclasts by binding on the surface receptor VEGFR2 [425]. Previous researchers have reported that the pre-treatment of the ceramic scaffold with RANKL induces osteoclastogenesis and expedites degradation [361].

It is intriguing to think about the mechanism by which the MSC-seeded dense collagen scaffold turned into bone callus. According to the histological assessment of the MSC-seeded dense collagen scaffolds cultured *in vitro*, the MSC started to exhibit osteoblastic differentiation on Day 5, mineralized the extracellular collagenous matrix on Day 10, and underwent apoptotic death on Day 15 (Figure 5.2). However, active osteoblasts and osteoclasts were populating the *in vivo* transplanted MSC-seeded dense collagen scaffolds even though they were mineralized extensively on Day 28 after surgery (Figure 5.5). The mineralized MSC-seeded dense collagen scaffold also functioned as an osteoconductive scaffold on which endogenous osteoblasts could easily attach and deposit extracellular bone matrix to contribute to more bone callus formation. As a result, the osteoclasts encroached on the vicinity of the transplanted collagen scaffold under chemotaxis. The osteoclasts degraded the transplanted collagen scaffold by secreting cathepsins, induced the formation of a multicellular unit, and remodeled the bone callus formed within the defect. Although the seeded MSC could differentiate into hypertrophic chondrocytes that initiate

the mineralization of the collagen scaffold, it is more likely that the MSC differentiated into osteoblasts directly by a mechanism similar to intramembranous ossification. As the animal model used in my thesis work abrogated micro-motion at the bone lesion, the transplanted MSC-seeded dense collagen scaffolds were not subjected to the mechanical stimuli favoring chondrogenesis [191]. However, for this model, it is worthwhile to evaluate the chondrogeneis of the transplanted collagen scaffold by specific histological staining. It would be more interesting to monitor both the intramemebranous and endochondral ossification occurring in the MSC-seeded dense collagen scaffold by using an animal model having micro-motion at the fracture site.

The MSC injected into the murine femoral canal to improve osseointegration were transplanted without *in vitro* osteoblastic induction and other modifications; whereas the MSC-seeded dense collagen scaffold was cultured in an osteogenic medium before *in vivo* transplantation. This transplantation protocol was based on the results of our *in vitro* studies that showed the capacities of undifferentiated MSC to adhere and differentiate on the titanium surface, as well as the timing at which the MSC-seeded collagen scaffold possessed maximal cellularity with the initiation of osteoblastic differentiation. The efficacy of the *in vitro* modifications of MSC prior to transplantation is open to debate [426]. An interesting study has indicated that the constructs composed of MSC and tissue culture plates led to an endochondral ossification when undergoing chondrogenic pre-induction, but intramembranous ossification without pre-induction [219]. Other studies have reported that the engineered bone construct seeded with osteogenic-induced MSC resulted in more bone regeneration than those with non-differentiated MSC [427]. Some researchers have suggested that a short period of pre-culture aiming at triggering the osteogenic differentiation but not completing the osteoblastic maturation

can lead to the largest amount of bone formation [428]. In this context, the *in vitro* treatment of MSC with recombinant growth factors or cytokines—which are pivotal to bone development or fracture healing, such as BMPs and FGFs—might be the best solution to optimize the proliferative and differentiation properties of the osteoprogenitor cells for bone tissue engineering [120]. Due to the remarkable variations associated with cell isolation, experiment design, animal models and scaffold usage, and the application of growth factors, great barriers were imposed for efficient data comparison among the different research groups.

While the application of dense collagen gel is still being investigated on the bench, the collagen sponge already has been translated to the bedside and approved for wound covering and hemostasis in practice [383 409]. Since it is a porous construct, the functionality of the collagen sponge has been studied by numerous research groups by using various growth factors including BMP and VEGF [286, 414]. By using the EDC chemistry described in Chapter VI, a collagen sponge disc measuring 8mm in diameter can covalently immobilize 97.2±8 ng VEGF, which was measured indirectly by subtracting the VEGF in washing solution, quantified by ELISA, from the starting amount added to the reaction system [383]. The MSC-seeded collagen sponges with the VEGF covalently tethered by using EDC chemistry significantly expedited the healing of critical-sized rat mandible defects compared to those sponges without the VEGF. Tethering VEGF with a collagen scaffold not only obviates the adverse effects associated with the use of a supra-physiological concentration in a single bolus injection, such as vascular leakage [145], but also avoids a second invasive procedure that increases the chances of infection. The prolonged and sustained release of VEGF over time that is associated with this immobilization methodology could be particularly beneficial in scenarios in which a long healing time is required, for example, critical-sized bone defects and fracture nonunion. Given the fact that most

critical-sized bone defect models are carried out with larger-scaled animal species, rats were used in the study to evaluate the effects of VEGF covalently tethered in a collagen sponge. The establishment of quantitative algorithms that can indicate the optimal dosage of VEGF for bone defects within specific parameters is valuable when translating to clinical practice.

7.2. Vascularized bone tissue engineering

The importance of the coupling of angiogenesis and osteogenesis cannot be exaggerated. Some researchers have implied that VEGF plays a role in both intramembranous and endochondral ossification at the early and late stages of bone healing [51]. VEGF can be secreted by multiple skeletal cells, including chondrocytes, osteoblasts, and osteoclasts [295], and it exerts its angiogenic effects in multiple ways. Primarily, VEGF is chemotactic and mitogenic to the EC harvested from arteries and veins [310]. VEGF is capable of increasing the survival of EC, enhancing vascular permeability, and inducing vasodilation [310]. Researchers believe that the profound bioactivities of VEGF are triggered by its binding to and activation of two tyrosine kinase receptors—VEGFR-1 (Flt-1) and VEGFR-2 (KDR/Flk-1)—as a dimer [310]. Both of these receptors are involved in the physiological and pathological vascularization process; however, VEFGR-2 is the main mediator of the signalling cascade in EC [310]. VEGFR-2, together with its co-receptor neurophilin-1 (NRP-1), is expressed in an endothelial tip, which induces the different biological responses of stalk and phalanx cells [429]. In brief, the tip cells are induced to migrate and branch, whereas the stalk cells are committed to mitogenic activity, which preserves the stalk phenotype. After the vascular branching is finished, the VEGF maintained at a low level is pivotal for the survival of the quiescent phalanx cells and vascular homeostasis [429]. A sufficient angiogenesis dictates the survival of the implanted construct and expedites the incorporation of the engineered bone graft with the surrounding tissue [430]. The newly formed blood vessels also

serve as conduits for osteogenic cells and osteoinductive molecules [431]. The angiogenesis is dictated by several signalling molecules, which can be explored as therapeutic targets to improve vascular engineering.

The presence of the VEGF receptors found in various kinds of cells besides EC implies that VEGF influences the migration, proliferation, differentiation, survival, and other bioactivities of many non-EC, including monocytes, hematopoietic stem cells, osteoclasts, and osteoblasts [58 432 433]. The nonvascular effects of VEGF on skeletal cells were mediated by means of paracrine, autocrine, and intracine mechanisms [51 50]. Although not fully illustrated, the nonvascular effects of VEGF were implied to be involved in skeletogenesis and bone homeostasis [50]. Researchers found that VEGF enhanced the biological effects of BMPs, which led to expedited MSC recruitment, prolonged cell viability, increased cartilage formation, and enhanced ossification [334].

In addition to VEGF, other angiogenic factors have been explored for engineering vasculature for expedited tissue regeneration. Angiopoietins are ligands for the endothelial cell receptor kinase TIE, and they play a pivotal role in initiating vascular sprouting [434]. PDGF and TGF-β are the most important molecular cues for recruiting pericytes and vascular smooth muscle cells, generating ECMs, and the final remodelling of newly formed vessels [328 295]. The hypoxia-induced factor 1α (HIF1α) signalling pathway has drawn increasing attention, since its expression is upregulated in response to hypoxia, which leads to an increased production of BMPs and VEGF [53].

The immobilization of angiogenic factors is especially promising for functionalizing biopolymers, including natural extracellular matrix molecules and synthetic polymeric materials

[435] [436]. In addition to the EDC chemistry we used for the covalent immobilization of VEGF, the pre-treatment of the collagen scaffold with other cross linkers, including heparin and SS-PEG-SS, was used to render the scaffold binding moieties to VEGF so that VEGF could be coated on the collagen scaffolds [321, 437]. The interactions between heparin and the angiogenic factors were non-covalent and more dependent on the electrostatic and hydrogen bond forces. Similarly, other angiogenic factors—such as PDGF and the biomaterial scaffolds other than collagen matrices, such as the demineralized bone matrix (DBM) and fibrin gel—can be coupled together by using heparin as the linker [438] [439] [440]. Alternatively, binding domains can be formed in the angiogenic factors by generating fusion proteins. Researchers found that the hepatocyte growth factor was bound to the collagen matrix following fusion with a collagen binding domain, which led to increased vascularization [441]. Also, VEGF can be directly incorporated as a bulk loading into various scaffolds, including collagen, PLGA, alginate, and others [431]. Researchers have reported that the degree of neovascularization is dependent on the dosage of VEGF delivered [442]. The various methods used to immobilize angiogenic factors to scaffolds, as mentioned previously, together with bolus injection, should be further compared with pharmacodynamics effects, and the pharmacokinetic curve should be carefully plotted to offer insight into the most cost efficient delivery strategy.

Multiple engineering technologies were developed to functionalize scaffolds to address the issues of angiogenesis. The creation of micro-patterning that leads to the formation of channels, grooves, and pores within the scaffold is feasible by utilizing the currently available technologies [443]. By using this strategy, flow regimes and cell alignment can be controlled [444]. The adhesion molecules present in the extracellular matrix, like RGD-peptide and fibronectin, can be incorporated into the scaffold with concentrations or surface-density gradients to guide

the proper growth and branching of newly formed blood vessels [445, 446]. Computer-aided 3D printing technology enables the precise engineering of internal and external architecture with a controlled spatial deposition of different cells and growth factors [403].

The co-culturing of EC or EPC with MSC is another strategy that can be used to promote the formation of blood vessel networks and augment new bone formation [402 447]. Multicellular spheroids containing endothelial cells are able to generate capillary sprouts *in vitro* in the presence of fibroblasts or when supplemented with VEGF and bFGF [448]. In addition, even a simple mixing culture of different tissue-specific cells in a biopolymer scaffold induces the formation of tubular structures [263 449]. The multiple mechanisms that underlie blood vessel formation in co-culture include cell-cell contact signalling, the production of cytokines and growth factors, and the neo-synthesis of ECMs [450]. The quantity of required cells for specific tissue is yet to be determined. Additionally, the anatomical tubular structure does not guarantee functional perfusion, and some researchers have found that the *in vitro* formed blood vessels contribute little to cell survival [447]. As such, a further exploration of the functional anastomosis between *in vitro* engineered blood vessels and the recipient host vessels is warranted. Other emerging technologies—such as microelectromechanical systems (MEMS), the modular assembly of endothelial cells, and *in vivo* vascular engineering by A-V loop or polysurgery—also have shown promise as alternative methodologies for engineering vascularized bone grafts [450].

Alongside the extensive exploration of vascularized tissue engineering is the great demand of robust experimental modality for quantitative 3D vasculature evaluation. The microCT scanning of enhanced vasculature provides the possibility of quantifying vascular volume. In this context, the commonly used agents for vascular perfusion and enhancement

include $BaSO_4$, lead chromate-loaded silicon, and polyurethane-based casting resin [451] [452]. With respect to quantifying vascular volume and density, the main disadvantage of lead chromate-loaded silicon is its comparable x-ray attenuation to bone, which necessitates decalcification before microCT imaging. [453]. $BaSO_4$ possesses a higher x-ray attenuation, which distinguishes blood vessels from bone structure in the CT images of undecalcified samples, and enables vascular quantification without decalcification [399]. In addition to yielding images with better quality, the $BaSO_4$ infusion is compatible with procedures used in bone histochemical staining and immunohistochemical staining for blood vessels [453].

7.3. Future research on MSC in bone tissue engineering

Approximately 50 clinical trials are being undertaken with a goal to evaluate the efficacy of MSC transplantation for skeletal reconstruction (http://clinicaltrials.gov). In the absence of high-leveled evidence about the efficacy and safety of MSC, robust recommendations cannot be made as to the indications and protocols of MSC usage. Indeed, the attempts to apply a cell-based bone augmentation strategy in clinical settings goes back at least two decades when the approach of injecting bone marrow preparations percutaneously into the defective bone lesion was introduced [454]. Currently, the more popular approach for delivering expanded MSC or fresh BMA is to pre-seed them into biomaterial scaffolds before they are transplanted *in vivo*. Ceramic scaffolds, including TCP and HA, were frequently used to carry the transplanted MSC because of their excellent osteoconductive and mechanical properties [21] [392]. Researchers have found that allograft seeded with expended MSC enhances both osteogenesis and osseointegration [455] [397]. Encouraging results with a high union rate and abundant callus formation were obtained in a limited number of clinical trials using BMAs and expanded MSC [219]; in addition, the indispensable contribution made by the transplanted BMA and MSC was highlighted by the

correlation between the density of transplanted cells and the volume of mineral callus [211]. To achieve sufficient cell density in the lesions, the local delivery of MSC is the preferred rout in bone tissue engineering, although the systematic administration of MSC enabled the targeting of injured tissues, which led to the studies on their potential for alleviating autoimmune diseases and healing necrotic myocardium [235].

Although the osteoprogenitors evaluated in these clinical trials were exclusively autogenous, the results I obtained during my PhD work, together with the current of preclinical data from other research groups, imply that allogeneic MSC can cause comparable outcomes to autogenous MSC even in immune competent recipients [456] [457]. Allogeneic MSC enhanced bone regeneration around the intramedullary implant in recipients irradiated only in the hind limbs and in the femoral defect in the presence of VEGF. The immune system in both models were actually intact. The immune privilege embodied in MSC also raised a great interest in the application of allogeneic MSC in tissue engineering, which not only would render the cell-based engineered bone constructs ready-for-use but also abrogate the negative effects that the systemic conditions of patients could possibly have on the autogenous MSC. For example, aging and chronic HBV infection became known systemic factors that impair the proliferation capacity of MSC [458]. Interestingly, the MSC extract from patients with atrophic nonunion proliferate at a lower rate, whereas the extract from multiple-trauma patients proliferates faster [459]. The decreasing osteogenic potential that accompanies the aging process is of concern with respect to the adequacy of a universal application of autogenous MSC in bone reconstruction, especially in aging populations who are complicated with systemic abnormalities and poised to benefit most from a cell-based bone repair strategy[460].

Some have suggested that allogeneic MSC can be used for transplantation without the ablative conditioning that is routinely done in hematopoietic stem cell transplantation [235] [236]. Indeed, several researchers have reported their successful experience with using xenogenous MSC for tissue repair in experimental models [237] [238]. The immune privilege embodied in MSC is attributed to the secreted immunomodulatory factors—such as IL-10—expressed on their surface, as well as the absence of antigenic molecules, such as HLA class II histocompatibility antigen [239] [240]. Furthermore, the majority of osteoblasts derived from the transplanted MSC only survive for a short period of time *in vivo* before being surrounded by minerals and undergoing apoptosis with a minority of osteoblasts being embedded in the newly formed bone structure as osteocytes that cannot impose a persistent immunological threat [241]. Some researchers have implied that the production of bone by the transplanted MSC is not as important as their secreted growth factors or cytokines that improve the microenvironments and exert trophic effects on the endogenous cells [235].

The plausibility of using allogeneic MSC for local bone reconstruction was further supported by the outcome of previous clinical trials that tested allogeneic MSC as a systemic administrated therapeutics for osteogenesis imperfecta (OI) [275]. The systemic delivery of allogeneic MSC was able to accelerate bone growth in a small cohort of patients with OI without causing the adverse effects that have been observed in both cohorts of healthy volunteers and patients with OI [461]. Nevertheless, the critical-sized bone defect in the rat mandible did not show improved healing when treated with collagen sponges seeded with mice MSC alone. Indeed, the efficacy of the xenogenous MSC in bone reconstruction is more ambiguous due to the variations of outcomes reported. For example, human MSC (hMSC) loaded on a poly-caprolactam-PCL-TCP scaffold healed the critical-sized defect made in rats' femurs [462]; in contrast, hMSC failed to

form as much bone as autogenous MSC when loaded in calcium-deficient hydroxyapatite and transplanted into the critical-sized defects in rabbits' ulnas [463].

Although successful experience was gained from harvesting and cryopreserving MSC at a laboratory scale, the establishment of an MSC bank at an industrial scale is still in its infancy [464]. Originally discovered in bone marrow [465], MSC have been isolated from nearly all the mesenchymal tissues and some non-mesenchymal tissues with different frequency, propensities to differentiate, and cell surface markers. Bone marrow-derived MSC (BM-MSC) display the greatest osteoblastic differentiation [466], but it is limited by the low frequency in bone marrow with approximately 1 MSC/10^4 mononuclear cells at birth, which decrease to 1 MSC/2×10^6 mononuclear cells in 80-year-old individuals [467]. The newly developed reamer/irrigator/aspirator system enables the harvest of larger amount of MSC together with morsellized autogenous bone during orthopedic surgery, and provides a possibility of establishing an allogeneic MSC bank for bone engineering [468].

Other tissues containing MSC with higher frequency are worth investigating for their potential as cell sources for bone engineering. Adipose tissue and umbilical cords appear to be promising alternatives to bone marrow because a large quantity of MSC can be easily harvested from these tissues [469]. Although inferior to BM-MSC in promoting chondrogenesis and osteogenesis [466], adipose-derived stem cell (ADSC) do not display an obvious age-related decline in proliferation as those derived from bone marrow [470]. Harvesting MSC from umbilical cord and placenta (UCSC) is totally non-invasive and can be performed at low cost. Similar to ADSC, UCSC cannot compete with those from bone marrow in osteogenic potential; nevertheless, they are superior to both adipose and bone marrow-derived MSC in their proliferative capacity [471], lower immunogenicity [422], and stronger ECM synthesis [472]. The

presence of MSC or osteoprogenitor cells in peripheral circulation is supported flow cytometry that has demonstrated that 1% of the circulating mononuclear cells are positive for both alkaline phosphatase (ALP) and osteocalcin (OCN) [473]. Some researchers have suggested that the age-related decline in the number of circulating osteoprogenitor cells contributes to impaired fracture healing in the elderly [473]. The newly discovered multipotent stem cells with both endothelial cell phenotypes and osteogenic capacity in the peripheral blood may be or become another potentially novel option for cell-assisted skeletal reconstruction [474]. The MSC used in my thesis work were isolated from total bone marrow cells by adherence to plastic TCP. Unlike hematopoietic stem cells (HSC), MSC do not possess a specific expression profile of surface markers or antigens except for the set of "conventional markers" as mentioned previously [223]. It is paramount to acknowledge the phenotypic and functional heterogeneity of the MSC utilized in preclinical and clinical studies, even though attempts have been made to purify the MSC by positive and/or negative selection with fluorescence-activated cell sorting (FACS) or magnetic bead sorting techniques that recognize the putative markers of MSC—including CD29, CD44, and STRO-1 [475] or those of non-MSC, including CD45, CD31, and CD34—to exclude hematopoietic and endothelial lineages [476] [477]. This heterogeneity of MSC was noted in conventional polyclonal culture more than one decade ago; actually, only subsets of MSC are capable of tri-lineage differentiation into chondrocytes, osteoblasts, and adipocytes while others are more committed to immunomodulatory activities or to supporting neural and hematopoietic cells [478]. As such, recognizing the function-associated markers is of great value in terms of purifying the specific subset of MSC with desired function and increasing the efficacy of MSC-based therapy. The recognized markers for functionally distinct subgroups of MSC include CD146, CD56, CD271, MSCA-1, Frizzled-9, and Stro-1 [478]. CD146, for example, is the marker

for the multipotent human MSC that maintains skeletal homeostasis by differentiating into osteoblasts, which reconstitutes the microenvironment for hematopoiesis [479]. CD271+ MSC are more committed to chondrogenic differentiation and immunomodulation [480] while Stro-1 enriched MSC are more clonogenic and proliferative [481]. Clonal functional assay in combination with surface marker arrays provide a viable approach for defining the functional MSC subgroups [478]. However, caution should be taken when translating the knowledge obtained from the studies on rodents to patients because of the considerable difference in the expression levels of markers between human and rodent MSC, given the same functionality.

The study published by Hernigou et al. in 2005 highlighted the paramount role of the number of osteoprogenitors by revealing the minimal MSC concentration of 1000/ml to achieve bony union in a cohort of 60 patients with tibia nonunion treated by percutaneous BMA injection [211]. However, the demand for the large number of MSC in certain clinical scenarios together with the limitations of the current harvesting technologies frequently necessitates the *in vitro* propagation of MSC. Researchers have shown that *in vitro* culture can lead to higher risk of infection and increased immunogenicity through exposure to animal serum products [234]. In addition, the *in vitro* propagation of MSC with extensive sub-cultivation could harm their therapeutic potency because of the reduced expression of adhesion molecules and surface receptors, lowered responsiveness to signalling molecules, impaired differentiation capacity, increased senescence and apoptosis, and enhanced genetic instability and tumorigenicity [482, 483] [484] [485]. One of the promising solutions to address the shortcomings of traditional culture is a bioreactor, a 3D culture system with more efficient mass transportation and mechanical stimuli[212]. Several types of bioreactors have been developed, including a spinner flask, a rotating-wall vessel bioreactor, a concentric cylinder bioreactor, and a perfusion bioreactor.

Although considered to be a superior strategy for enhancing the proliferation of MSC without altering their phenotype and differentiation potential, the application of bioreactors in regenerative medicine needs further investigation.

Cell labelling technologies that track the fate of transplanted cells will be broadly used in regenerative medicine employing stem cells [486]. However, convincing evidence is needed to demonstrate the cause-effect relationship between the transplantation of stem cells/osteoprogenitors and bone regeneration. Furthermore, cell tagging technology opens the way for establishing the quantitative correlations between the utilized cell quantity and regenerated bone mass under different circumstances, which will be of great value for guiding the clinical use of stem cells for bone reconstruction.

Appendix I: Methodology of Bone Tissue Engineering Research

As a pivotal part of bone tissue engineering (BTE) research, the preclinical study should provide an honest evaluation of the therapeutic potentials of the engineered bone constructs and profound insights into the strategies for improving their *in vivo* performance. Therefore, the validity of the methodologies used in BTE preclinical research forms the premier of reaching any valuable conclusion.

Animal models for orthotopic bone regeneration

The preclinical model using species other than human beings, either small or large, serves as a useful tool for *in vivo* BTE research. First, the task of surgical bone reconstruction can be simulated on experimental animals by generating bone defects modeling similar to those used in clinical scenarios, fixating bony fragments using a fixation instrument comparable to those used in clinical applications, and implanting the to-be-studied novel BTE therapeutics. In this respect, the selection of animal species is relevant in that surgical interventions should be made to ensure that the physiological processes of bone repair and the pathogenesis of nonunion are similar between human patients and animal models; also, the biomechanical stress that is imposed on the reconstruction sites of the animal models and clinical cases should be comparable. Bone defects of various scales, including non-critical-sized and critical-sized, can be made to model different categories of clinical problems and should be chosen based on both hypothesis and technical plausibility.

In my PhD study, I developed several surgically-modified rodent models to evaluate potential intraosseous therapeutics. A drill-hole model was made in the cortical bone of the

murine femur by using a 22G needle (Fig A.1). This model is advantageous in the simplicity of its surgical procedure and the shorter post-operative time required for the presence of the detectable biological difference. Three-dimensional microCT reconstruction showed that the drill hole without bone immediately after drilling had been filled with abundant callus at post-operative 2 weeks (Fig A.1 c). Quantitative analysis of microCT images (Fig A.1 d) combined with qualitative histological assessments (Fig A.1 e, f, and g) is effective for demonstrating the distribution of mineral and non-mineral components (Fig A.1 e), as well as the ALP activities of osteoblasts (Fig A.1 f) and TRAP activities of osteoclasts (Fig A.1 g). Nevertheless, when I used this model to test the MSC-seeded dense collagen scaffold described in Chapter IV, I found that improper handling of the collagen scaffold, such as unavoidable excessive squeezing and numerous trimming, inevitably introduced other uncontrollable artefacts to the result. Consequently, I discarded the drill-hole model for this project and established a new model by drilling three holes in a row and bridging them together into a larger window defect that enabled a gentle implantation of the collagen scaffold in a consistent manner.

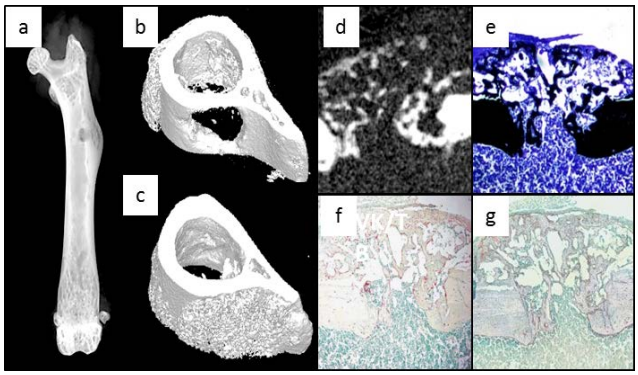

Figure A.1 Drill-hole model for BTE evaluation: A hole was made on the cortex of mouse femur by drilling with a 22G needle (a). MicroCT 3D reconstruction of the drill hole on Day 0 showing no callus (b) and postoperative day 14 showing abundant callus around the hole (c). Quantification of bone volume can be performed on the microCT images (d). Serial sections are used for Von Kossa & Toluidine Blue staining (e) to show mineralized and unmineralized components of callus, ALP staining (f) for osteoblasts and TRAP staining (g) for osteoclasts.

Although the window defect is larger and failed to heal at post-operative 4 weeks, I hesitate to categorize it as critical-sized defect due to the relative shorter post-operative period compared to other literature [64]. The defect measuring 4mm made in the rat mandible used in the study, as described in Chapter VI, is a putative model of critical-sized bone defect, which remained unhealed at post-operative 12 weeks [418 65]. Similarly, a critical-sized defect made in the skull, which can be used as a model for craniofacial surgery, has been reported and replicated in our lab as well (Fig A.2).

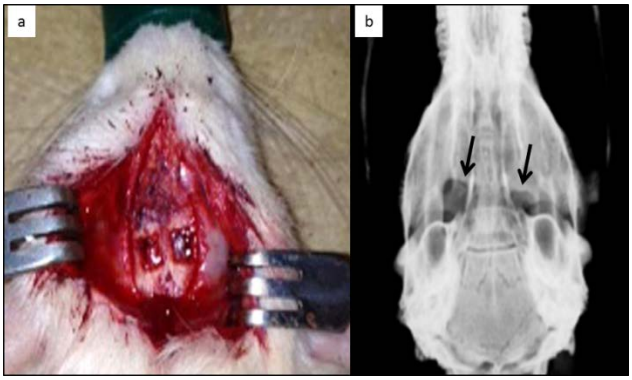

Figure A.2 Window defect on the rat cranium: Window defects measured at 3mm x 4mm drilled on the rat cranium (a) are shown as rectangle hyprodense areas on the plain x-ray (black arrows in b).

Another type of critical-sized defect that we established was the segmental defect generated in the rat femur, which was stabilized with an internal fixation apparatus consisting of a self-designed custom-made polyethylene plate (Fig A.3a) and K wires (Fig A.3b). A bone segment measuring 5mm was removed from the femoral diaphysis to form a fracture gap as demonstrated in the pictures taken intra-operatively (Fig A.3c) and post-operatively (Fig A.3d). As predicted, radiography did not show an obvious difference in the size of the segmental defect between the operation day (Fig A.3e) and post-operative 12 weeks (Fig A.3f). This model has been used to test the novel Ti foam fabricated by the National Research Council of Canada. The Ti foam customized according to our design requirements with a specific dimension and geometrical shape was inserted into the fracture gap to replace the removed segment (Fig A.3g) and induced bone formation within the defect at post-operative 12 weeks (Fig A.3h) in contrast to those defects that did not receive an intervention.

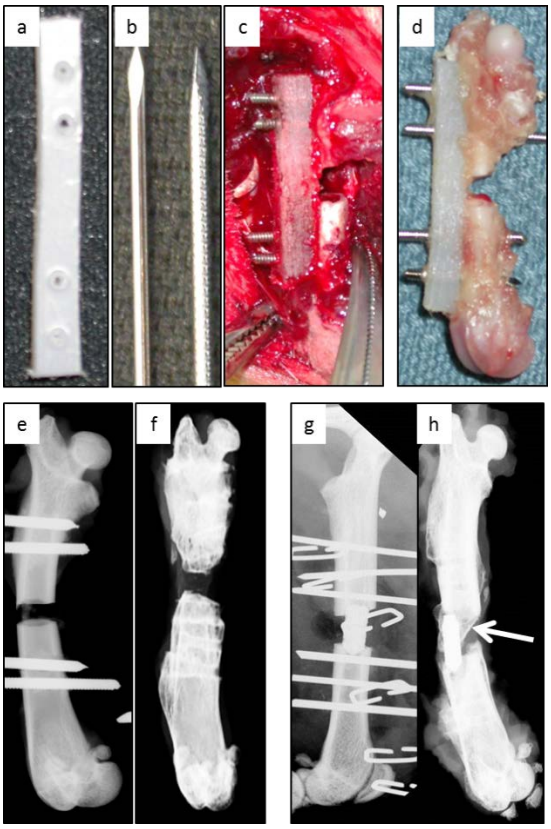

Figure A.3 Critical-sized segmental defect in rat femur: Custom made polyethylene plate (a) and Kirshner wires (b) are used to fix the bone fragments leaving 5mm gap unclosed (c and d). The 5mm fracture gap shown on plain x-ray taken at Day 0 (e) failed to heal by postoperative 12 weeks (f). Given a Ti foam implanted into the same gap at day 0 (g), bone regeneration was observed around the Ti foam at postoperative 12 weeks (arrow in h).

The critical-sized defect models mentioned above failed to heal spontaneously due to the significant loss of bone that disables the physiological healing mechanisms to repair the lesion completely. However, the recalcitrant defect seen in fracture nonunion is not necessarily that large in size and more attributable to not only mechanical instability, but also the disruption in the healing biological cascade by the lack of certain biological entities. Therefore, targeting—such as sub-lethal irradiation, chemotherapy, destructing periosteum, and so on—the impairment of the biological entities that are pivotal to fracture healing has been used by several researchers to generate animal models for fracture nonunion [63 66 487]. We developed an original minipig model of fracture nonunion of scaphoid (Fig A.4a) by staged surgical modifications. To facilitate the comparison, different surgical operations were done in the LEFT and RIGHT forelimbs of the minipig. On the LEFT side, the radiocarpal bone was osteotomized in the center with a 3mm bone segment removed and fixed with a Ti screw on day 0 (Fig A.4b and c); on the RIGHT side, the osteotomy with a 3mm bone segment was removed, and the defect was filled with a dense collagen gel on day 0 (Fig A.4d and e), followed by 6 weeks of unrestricted weight bearing. At post-operative 7 weeks, the RIGHT radiocarpal bone was cleared of the inserted collagen gel and fixed with the same Ti screw as used in the LEFT side (Fig A.4f and g). Twelve weeks following the second staged surgery, radiographies showed the defect in the LEFT radiocarpal was bridged completed (Fig A.4h), whereas not as much bone regeneration was observed in the RIGHT defect (Fig A.4i). The same conclusion was reached with the 3D microCT reconstruction that showed a complete healing of the defect on the LEFT side (Fig A.4j) and nonunion on the RIGHT side (Fig A.4k), which provided more evidence of the validity of this nonunion model generated by staged surgical operations.

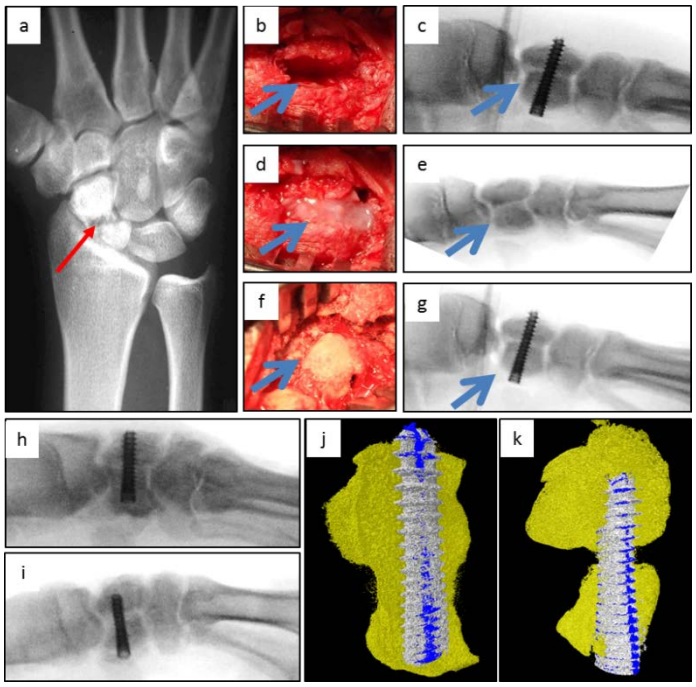

Figure A.4 Minipig model of scaphoid fracture nonunion: Two approaches were explored to develop the minipig model of scaphoid fracture nonunion as seen in patient (a). The first approach consists of generation of 3mm segmental defect in the radiocarpal bone (b) and internal fixation with cannulated screw (c). The second approach using staged surgeries consists of generation of 3mm segmental defect followed by dense collagen gel interposition (d) at Day 0 (e) and collagen removal (f) with internal fixation with cannulated screw (g) at postoperative 6 weeks. At postoperative 19 weeks the fracture gap generated by the first approach healed completely as proved by plain x-ray (h) and microCT 3D reconstruction (j) while the fracture gap generated by the second approach failed to heal as shown in plain x-ray (i) and microCT 3D reconstruction (k).

Larger species are superior to smaller species for preclinical studies of BTE therapeutics because the former share more similarities with humans in their biological and mechanical features [69]. However, rodent models are still being used extensively in BTE research. First, the accumulated knowledge on mouse and rat genomes enables the generation of genetically engineered rodent models that exhibit abnormal bone phenotypes that resemble different bone diseases [71], and thus provide unique models to study tissue engineering strategies to improve bone regeneration under specific circumstances. As described in Chapter II, the use of the C3H FGFR3-/- mouse model was the basis for our conclusion that was applicable to those patients with osteopenia—that MSC transplantation can promote the osseointegration of an intramedullary Titanium implant. Inbred rodents are characterized by low cost, easy access, exhaustive characterization, and simplicity of pharmacological or physical conditioning [68, 70]; therefore, they are ideal candidates for preclinical models for proof-of-concept. In this context, the ovariectomized (OVX) rat model is a US FDA approved model for the evaluation of new therapeutic agents for postmenopausal osteoporosis. An aged murine model was used in the project described in Chapter V to evaluate the MSC-seeded dense collagen scaffold to promote bone healing in the elderly; nevertheless, further evaluation with respect to the pharmacological treatment of osteoporosis, which is common among the elderly, is worthwhile, and the aged murine model administrated with an anti-osteoporosis agent, such as bisphosphonate, is promising for providing valuable information at lower cost.

Skeletal phenotyping

The phenotyping of bone regeneration and repair shares a great similarity to skeleton phenotyping, which can be divided into three levels (Fig A.5). The primary stage of phenotypic analysis mainly consists of plain X-ray imaging that enables qualitative judgement of the degree of bone formation; as well as microCT analysis that allows for a visualization of bone regeneration from different perspectives, 3D reconstruction and, more importantly, objective quantification of bone volume and architecture. Secondary screening is performed on un-decalcified bone samples to lend further evidence of the structure of the bone matrix and cellular activities, which complements the quantitative results obtained from microCT. The tertiary phenotyping requiring decalcified bone samples is at the molecular and genetic levels, and demands more sophisticated techniques. Experimental methodologies at this level often are needed to address the mechanisms that lead to final findings.

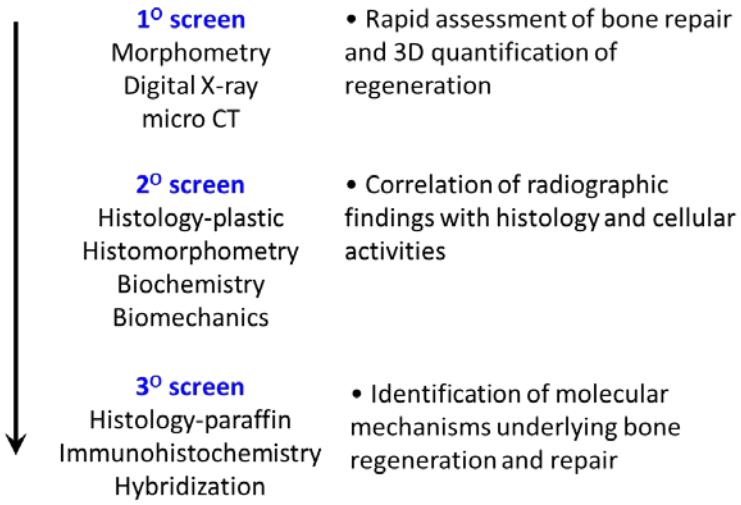

Figure A.5 Sequential skeletal phenotyping protocol used in the Bone Engineering Labs, MUHC.

Quantitative microCT analysis

The most essential component of microCT quantification is a proper definition of Region of Interest (ROI), the region in which the desired biological activities are supposed to occur and bone structure is to be quantified. The ROI can be defined by choosing a simple regular shape, such as circle or rectangle, which has been set in the analytic software, or by manual drawing any irregular shapes across all the slices in the dataset (Fig A.7). However, the innovative use of analytic software (CTAn) by composing a customized program to enable the computer to define the ROI automatically should be encouraged because, in addition to helping eliminate the inter-observer bias, it enables a precise outline of the region of bone regeneration across the multiple 2D slides of the dataset in a few minutes, no matter how irregular the shape of the region.

The delineation of ROI can be done based on the difference in greyscale as a result of different X-ray attenuation. This strategy is commonly useful for the study of the osseointegration of intraosseous implants that exhibit different densities of bone on radiography. An example shown here is the collaboration with NRC that required the quantification of the volume of the newly formed bone induced by the implanted Ti foam within the critical-sized fracture gap (Fig A.6). The newly formed bone induced by the Ti foam can be visualized on plain X-ray film (Fig A.6a), and the difference of the greyscale between the bone and the Ti implant can be seen in the 2D microCT images (Fig A.6b). By adjusting the greyscale segmentation threshold, the shadows of the implant and bone can be separated with ROI being defined only to cover the space occupied by either implant (Fig A.6c) or bone (Fig A.6e) automatically by the computer. As a consequence, both implant (Fig A.6d) or bone (Fig A.6f) can be quantitatively analyzed without interference from the other, which leads to the plausibility of assessing *in vivo* implant degradation as well as bone formation. A three-dimensional

reconstruction that differentiates the implant and bone can be done by combining the ROIs of bone and implant in the CTVol software. The cut in cross sectional plane (Fig A.6g) and longitudinal plane (Fig A.6h) of the 3D model clearly demonstrates the bone ongrowth as well as ingrowth, while the gross 3D model provides a wider perspective on the extent of bone growth onto the implant (Fig A.6i).

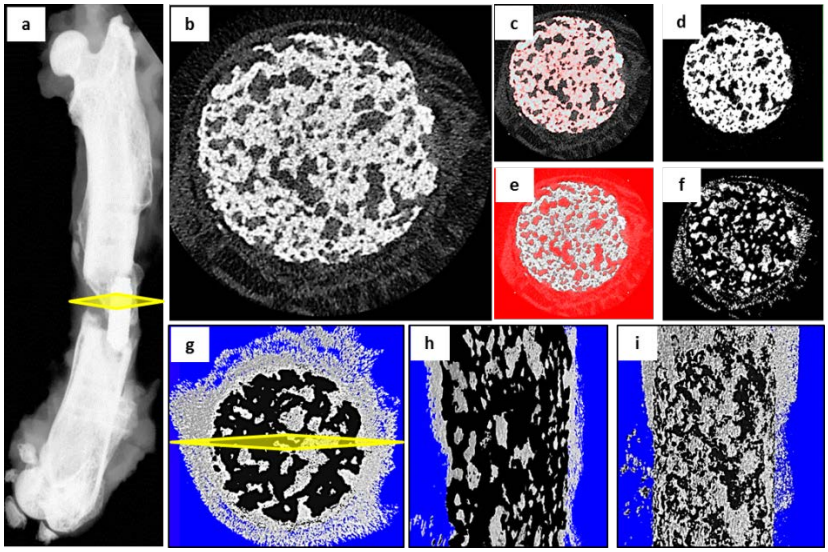

Figure A.6 Defining ROI by adjusting greyscale threshold for binarization: Ti foam implanted into critical-sized defect induced bone formation around the implant at postoperative 12 weeks (a). The cross sectional microCT image (b) corresponding to the level marked by the yellow diamond in (a) shows Ti foam with higher density and bone structure with lower density. By adjusting the threshold for binarization, one can define Region of Interest (ROI) (red in c and e) to include only either Ti implant (d) or bone (f) in order to segment one from the other. As such, quantification and 3D reconstruction can be performed. Transaxial view 3D reconstruction of the Ti foam at postoperative 12 weeks revealed bone formation (white in g) occurred both onto and into the implant (black in g). A sagittal or coronal cutting plane (yellow diamond in g) facilitates the visualization of bony ingrowth longitudinally (h). Similarly, 3D reconstruction from a sagittal or coronal perspective without cutting facilitates visualization of bony ongrowth longitudinally (i).

By exploiting the difference of bony morphological properties, ROI can be defined to demarcate certain specific anatomical zones for quantification. The trabecular structure of the callus in sharp contrast to the dense cortical bone makes the newly formed bone callus separable from the original host bone, which renders valuable information on the bone callus, including the volume, spatial extent, density, and connectivity to name only a few (Fig A.7a and b). The same strategy has been used to analyze the mineralization of the epiphyseal plate (Fig A.7c and d), the morphological properties of the cortical (Fig A.7f and g) and trabecular bone (Fig A.7h and i) in genetically mutant mice, and to quantify the subchondral (Fig A.7k) and trabecular (Fig A.7l) bony changes in the steroid-induced rat femoral head necrosis.

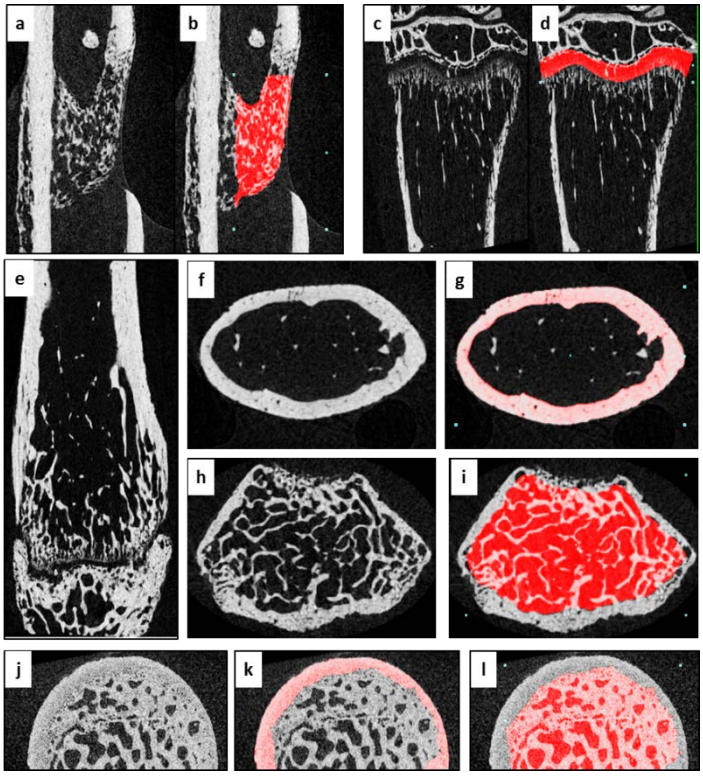

Figure A.7 Defining ROI by exploiting the difference of morphological properties: In case there is no difference of grey scale, the distinct morphological property of bone structure in ROI can be used to delineate ROIs that outline the anatomical structure precisely. This approach is especially useful in quantifying bone structure in an irregular-shaped region, such as bone callus formed during fracture healing (a and b), growth plate (c and d), diaphyseal cortical bone (f and g), metaphyseal trabecular bone (h and i), as well as subchondral and trabecular bone in femoral head (j, k and l).

$BaSO_4$ perfusion was used in my thesis work to evaluate the extent of vascularization around the critical-sized bone defects in the mandible, as shown in Chapter VI, as well as femoral diaphysis (Fig A.8d). The intra-abdominal aortic perfusion can enhance the vasculature distributed in the pelvis (Fig A.8a) and hind limbs (Fig A.8b, c) which can be indicated when studying the blood supply of femur head in osteonecrosis and vascularization around fracture nonunion. Compared to the vasculature around the intact femur (Fig A.8d), the vessels were remodeled and more densely distributed adjacent to the critical-sized bone defect at postoperative 3 months (Fig A.8e). The improvements of vascular imaging by microCT is largely dependent on the development of new biomaterials that are compatible with infusion procedure, display distinct x-ray attenuation from bone and coagulate within the blood vessels over a decent period of time. Besides, it is still unclear to what extent the vasculature is related to the bony pathological changes and fracture healing which renders limitations to the reliability of quantitative vascular analysis.

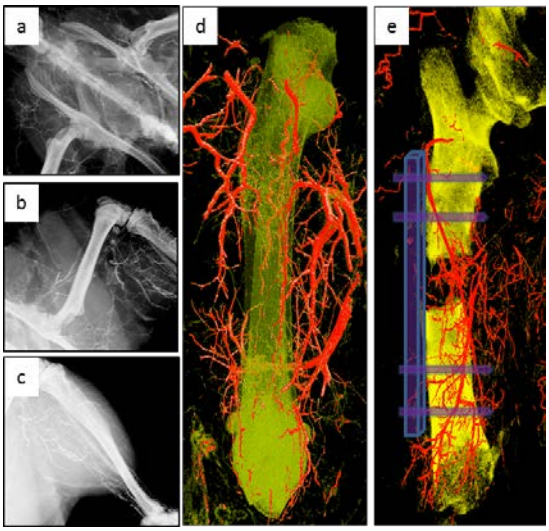

Figure A.8 MicroCT imaging of vasculature enhanced by $BaSO_4$ perfusion: Vasculature of the hind limbs of rat can be imaged by microCT following catheterizing and perfusing abdominal aorta with $BaSO_4$. The vasculature of adjacent to pelvic girdle (a), femur (b) and tibia (c) are then enhanced as shown in plain x-ray. MicroCT 3D reconstruction enables the visualization of the different vasculatures around an intact femur (d) and a critical-sized defect at postoperative 12 weeks (e).

Comparative histology

In addition to the 3D quantification by microCT, histology can be used to illustrate the biological activities that account for the response to BTE therapeutics. As a 2D imaging method, Histology is limited, so conclusions should only be drawn on the premise that the comparability of the histological results is guaranteed. First, efforts should be made to ensure that the histological sections obtained from the control and treatment groups are from similar anatomical locations that can be facilitated by controlling the sample direction during embedding and recognizing the spatial coordinates in relation to certain anatomical landmarks, such femoral trochanters and epiphyseal plate, during sectioning. In addition, the sectioning of histological samples can be directed by the microCT dataset that consists of thousands of 2D images from different perspectives so that the sections of the areas where meaningful biological activities are presumed to occur can be harvested without missing essential information in a timely manner. Last, after the site of interest is reached, attempts to harvest consecutive sections should be encouraged. Different staining of these consecutive sections can reveal the relationships or interactions among different biological entities, such as osteoblasts, osteoclasts, vessels, and mineralized bone matrix, which is exemplified by my work described in previous chapters (Fig 4.5, 5.7, 5.8, 6.3, 6.4).

Appendix II. References

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Appendix III. Collaborations

This section includes 4 publications I contributed as the second author druing my PhD study.

1) A novel biological agent, FGF18, was evaluated for its potential in improving osseointegration by using the same murine model of intra-femoral implant as dscribed in Chapter IV.

2) In a collaboration with colleagues at the veterinary school of Universite de Montreal (Saint-Hyacinthe, QC), the correlation between cartilage and bone pathologies was examined in the autopsy specimens of race-horses whose careers had been terminated due to osteoarthritis.

3) In a collaboration with colleagues at the Faculy of Dentistry of McGill University, the effect of arterial calcification on skeletal phenotype was studied by using MGP-/- murine model.

4) My expertise in small animal model development and skeletal phenotyping acquired during my graduate studies has led to a book chapter.