Craniomaxillofacial Bone Tissue Engineering – A Translational Approach

by

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In memory of my beloved Father,
to Gamal Taha Abushahba,
I dedicate this work,

ABSTRACT

Bone tissue engineering (BTE) has shown a great promise in providing the next generation medical bioimplants for treating bone defects. However, BTE faces many obstacles which need to be addressed for promoting translatability. The objective of this thesis work was to explore clinically translatable tissue engineering approaches for the management of craniomaxillofacial bone defects. The role of the employed cells has witnessed a critical turning point towards an increased appreciation of the cellular paracrine effects. This paracrine effect is mediated via secreted proteins and released membrane-bound vesicles called extracellular vesicles (EVs). For advancing our knowledge about the biological roles of EVs, we employed RNA sequencing to provide a comprehensive overview of the expression profiles of small non-coding transcripts carried by the EVs derived from human adipose tissue stromal/stem cells (AT-MSCs) and human pluripotent stem cells (hPSCs). Our findings revealed distinctive small non-coding RNA profiles from hPSCs and AT-MSCs EVs. The regulatory miRNAs of stem cells at cellular level are also present in their EVs, indicating an important regulatory role which is mediated via EVs.

Vascularization is the key challenge for BTE applications in large bone defects. The local delivery of growth factors leads to short lived effects. Small molecule chemicals feature alternative cost-effective bioactive agents with better stability. We assessed the ability of two small molecules; DMOG and baicalein, in triggering the proangiogenic secretome of AT-MSCs *in vitro*. Additionally, other effects, such as proliferation and osteogenic differentiation of AT-MSCs were assessed. DMOG and baicalein efficiently stabilized the hypoxia-inducible factor (HIF-1α) and upregulated proangiogenic cytokines, e.g., vascular endothelial growth factor (VEGF) and platelet-derived growth factor-BB (PDGF-BB) of AT-MSCs in normoxic conditions. These effects were further associated with upregulated stemness-related gene expression, slowed proliferation, and reduced osteogenic potential. Chemically-induced hypoxia maintained the stemness and self-renewal properties of AT-MSCs, while enhancing their proangiogenic potential.

The *in vivo* bioreactor (IVB) concept combines the potential of BTE and reconstructive surgery by employing the patient body for prefabricating new prevascularized tissues. Ideally, IVB should minimize the need for exogenous growth factors or cells and harness the native regenerative potential of employed tissues. Using acellular alloplastic bone blocks, we compared muscle-IVB with and without periosteal/pericranial grafts and flaps for prefabricating tissue engineered bone (TEB) flaps. We also assessed their functional outcomes in reconstructing a mandibular defect in an ovine model. The employment of vascularized periosteal flaps did result in more robust vascularization as compared to other IVB techniques. Both the periosteal grafts and periosteal flaps enhanced the performance of the prefabricated TEB flaps after transplantation into a mechanically stimulated bony microenvironment. However, more new bone formation and biomaterial remodeling was associated with the vascularized periosteal flaps.

ABSTRAKTI

Luukudosteknologiasta (BTE) odotetaan uuden sukupolven kudossiirteitä luukudosvaurioiden hoitoon. Tämän väitöskirjan tavoitteena oli tutkia kliinisesti sovellettavia kudosteknologisia lähestymistapoja kallon ja kasvojen luuvaurioiden korjaamiseksi. Solujen rooli kudosteknologiassa on muuttunut merkittävästi viime vuosikymmenellä kohdistaen tutkimuksen yhä enemmän solujen parakriinivaikutukseen, joka välittyy erittyvien proteiinien ja ekstrasellulaarivesikkelien (EV) kautta. Ymmärtääksemme ihmisen kantasoluista peräisin olevien EV:en roolin kudosteknologiassa, sekvensoimme näiden sisältämien pienten ei-koodaavien RNA-molekyylien ilmentymisprofiilit rasvakudoksen strooma-/kantasoluista (AT-MSC) ja pluripotenteista kantasoluista (hPSC). Totesimme selvät eroavaisuudet näiden välillä, osoittaen solukommunikaation etenevän myös EV-välitteisesti.

Vaskularisaatio on keskeinen haaste BTE-sovelluksissa suurissa luukudosvaurioissa ja on todettu, että paikallinen kasvutekijöiden annostelu vaikuttaa vain lyhytaikaisesti. Pienimolekyylisissä yhdisteissä on tarjolla vaihtoehtoisia kustannustehokkaita bioaktiivisia aineita, joista tutkimme kahden, dimetyylioksalyyliglysiinin (DMOG) ja baikaleiinin, vaikutusta AT-MSC:n proangiogeenisen sekretomiin. DMOG ja baikaleiini stabiloivat tehokkaasti hypoksiaa indusoivaa tekijää (HIF-1a) ja säätelivät proangiogeenisia sytokiineja, kuten verisuonen endoteelikasvutekijää (VEGF) ja verihiutalekasvutekijää (PDGF-BB), mikä osoitti kemiallisesti indusoidun hypoksian edistävän AT-MSC:n proangiogeenista potentiaalia.

In vivo bioreaktorikonseptin (IVB) tarkoituksena on hyödyntää potilaan omaa kehoa uudiskudosten verisuonittumisessa, poistaen paikallisesti annettujen kasvutekijöiden tai -solujen tarpeen hyödyntämällä kudosten luontaista uusiutumiskykyä. Tutkimuksessa testattiin lammasmallissa tehostetun luutumisen ja verisuonittumisen aikaansaamiseksi lihas- ja luukalvosiirteitä sekä -kielekkeitä alloplastisilla luusiirteillä. IVB-rakenteiden toiminnallisuus arvioitiin rekonstruoimalla näiden avulla leukakulman luuvaurioita. Verisuonittunut luukalvokieleke-IVB johti voimakkaimpaan verisuonittumiseen ja tehostettuun luuvaurion korjaantumiseen leukakulmassa verrattuna muihin IVB-tekniikoihin.

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LIST OF ORIGINAL PUBLICATIONS

This doctoral thesis is based on the following original studies (publications), which are referred to in the text by their Roman numerals (I-III):

I. Kaur S*, **Abu-Shahba AG***, Paananen RO, Hongisto H, Hiidenmaa H, Skottman H, Seppänen-Kaijansinkko R*, Mannerström B*. Small non-coding RNA landscape of extracellular vesicles from human stem cells. Sci Rep. 2018 Oct 19;8(1):15503. doi: 10.1038/s41598-018-33899-6. PMID: 30341351; PMCID: PMC6195565.

II. **Abu-Shahba AG**, Gebraad A, Kaur S, Paananen RO, Peltoniemi H, Seppänen-Kaijansinkko R, Mannerström B. Proangiogenic Hypoxia-Mimicking Agents Attenuate Osteogenic Potential of Adipose Stem/Stromal Cells. Tissue Eng Regen Med. 2020 Aug;17(4):477-493. doi: 10.1007/s13770-020-00259-3. Epub 2020 May 24. PMID: 32449039; PMCID: PMC7392999.

III. **Abu-Shahba AG**, Wilkman T, Kornilov R, Adam M, Salla KM, Lindén J, Lappalainen AK, Björkstrand R, Seppänen-Kaijansinkko R, Mannerström B. Periosteal flaps enhance prefabricated engineered bone reparative potential. *Submitted.*

* These authors contributed equally.

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Additional publications

List of additional publications not included in this thesis:

- Saad KA, **Abu-Shahba AG**, El-Drieny EA, Khedr MS, “Evaluation of the role of autogenous bone-marrow–derived mesenchymal stem cell transplantation for the repair of mandibular bone defects in rabbits”, *Journal of Cranio-Maxillofacial Surgery*, 43 (7): 1151-1160 (2015).

- Mannerström B, Kornilov R, **Abu-Shahba AG**, Chowdhury IM, Sinha S, Seppänen-Kaijansinkko R, Kaur S, “Epigenetic alterations in mesenchymal stem cells by osteosarcoma-derived extracellular vesicles”, *Epigenetics*, 14 (4): 352-364 (2019).

- Mannerström B, Paananen RO, **Abu-Shahba AG**, Moilanen J, Seppänen-Kaijansinkko R, Kaur S. “Extracellular small non-coding RNA contaminants in fetal bovine serum and serum-free media”, *Scientific Reports* 9, Article number: 5538 (2019).

- Dienel K, **Abu-Shahba AG***, Kornilov R*, Björkstrand R, Bochove B, Snäll J, Wilkman T, Mesimäki K, Meller A, Lindén J, Lappalainen AK, Partanen J, Seppälä J, Seppänen-Kaijansinkko R, Mannerström B. Manufacturing patient-specific bioimplants and reconstruction plates for mandibular defects: production workflow and *in vivo* large animal model study. *Submitted*.

- Adam M, Lindén J, Raekallio M, Mannerstörm B, **Abu-Shahba AG**, Seppäinen-Kaijansikko R, Meller A, Salla KM. Concentrations of vatinoxan and xylazine in plasma, cerebrospinal fluid and brain tissue following intravenous administration in sheep. *Submitted*.

- Adam M, Lindén J, Raekallio M, Meller A, Mannerstörm B, **Abu-Shahba AG**, Seppäinen-Kaijansikko R, Salla KM. Effects of vatinoxan on xylazine-induced pulmonary alterations in sheep. *Submitted*.

  * These authors contributed equally.

PERSONAL CONTRIBUTION

Publication I

I carried out adipose tissue-derived mesenchymal stem cells (AT-MSCs) isolation, characterization, and culturing. I performed western blotting and analysis of the data. I drafted and revised manuscript together with the other co-authors.

Publication II

I contributed to the conception and design of the study. I performed AT-MSCs isolation, characterization, and culturing. I carried out all the analyses. I interpreted the results with supervisors, drafted, and critically revised the manuscript with co-authors.

Publication III

I contributed to the conception, design of the study, coordination of collaborations, and performing the animal experiment. I assisted in the surgical phases, pre- and post-operative management, and in samples and data acquisition. I performed the analyses, interpreted the results, drafted, and critically revised the manuscript with other co-authors.

ABBREVIATIONS

3D	Three-dimensional
ACS	Absorbable collagen sponge
ALP	Alkaline phosphatase
AM	Additive manufacturing
ARS	Alizarin Red S stain
AT-MSCs	Adipose tissue derived mesenchymal stromal/stem cells
BLCs	Bone lining cells
BMP	Bone morphogenetic protein
BMSCs	Bone marrow mesenchymal stem/stromal cells
BMU	Basic multicellular unit
BRC	Bone remodeling compartment
BSP	Bone sialoprotein
BTE	Bone tissue engineering
CAD-CAM	Computer aided design-computer aided manufacturing
c-Fms	Colony stimulating factor-1 receptor
CSD	Critical-size defect
CSF-1	Colony stimulating factor-1
CT	Computed tomography
CTA	Computed tomography angiography
DBM	Demineralized bone matrix
Dlx5	Distal-less homeobox 5
DMOG	Dimethyloxalylglycine
DMP1	Dentine matrix protein 1
DMSO	Dimethyl sulfoxide
DO	Distraction osteogenesis
ECM	Extracellular matrix
EVs	Extracellular vesicles
FBS	Fetal bovine serum
FDA	United States Food and Drug Administration
FGF	Fibroblast growth factor
FIH	Factor Inhibiting HIF-1α
GA	General anesthesia
GMP	Good manufacture practice
GSK-3β	Glycogen synthase kinase 3 beta
HA	Hydroxyapatite
hESCs	Human embryonic stem cells
HIF	Hypoxia-inducible factor
hiPSC	Human induced pluripotent stem cells
hPSCs	Human pluripotent stem cells
HRE	Hypoxic response element
HSP 70	The 70 kilodalton heat shock proteins
ICAM1	Intercellular adhesion molecule 1
IF	Immunofluorescence
IGF	Insulin-like growth factors
IHC-P	Immunohistochemistry for paraffin embedded sections
ISCT	International Society for Cellular Therapy
IVB	*In vivo* bioreactor
MCP-1	The monocyte chemoattractant protein-1
M-CSF	Macrophage colony-stimulating factor
mi-RNA	Micro RNA
mRNA	Messenger RNA

MSCs	Mesenchymal stem/stromal cells
NABGs	Nonvascularized autologous bone grafts
ncRNA	non-coding RNA
NTA	Nanoparticle tracking analysis
OCN	Osteocalcin
OPG	Osteoprotegerin
Osx	Osterix
OXPHOS	Oxidative phosphorylation
PCL	Poly (ε-caprolactone)
PDGF	Platelet-derived growth factor
PEEK	Poly (ether-ether-ketone)
PGA	Poly (glycolic-acid)
PHD	Prolyl hydroxylase domain enzymes
PHIs	Prolyl hydroxylase inhibitors
PI3K	Phosphoinositide 3-kinase
PLA	Poly (lactic-acid)
PLGA	Poly (lactic-*co*-glycolic acid)
PPE	Porous polyethylene
PSI	Patient-specific implants
PTEN	Phosphatase and tensin homolog
PTH	Parathyroid hormone
PTMC	Poly (trimethylene carbonate)
qRT-PCR	Quantitative real-time polymerase chain reaction
RANK	Receptor activator of nuclear factor kappa-B
RANKL	Receptor activator of nuclear factor kappa-B ligand
RGD	Arginylglycylaspartic acid
rhBMPs	Recombinant human BMPs
RT	Room temperature
Runx2	Runt-related transcription factor 2
SCF	Stem cell factor
SLA	Stereolithography
SLS	Selective laser sintering
Sox9	SRY (sex determining region Y)-box 9
SR-PLA	Self-reinforced poly (lactic-acid)
TCP	Tricalcium phosphate
TEB	Tissue engineered bone
TEM	Transmission electron microscopy
TGF-β	Transforming growth factor beta
TSG101	Tumor susceptibility gene 101
VEGF	Vascular endothelial growth factor
WB	Western blotting
Wnt	Wingless-Int-1
β-catenin	Catenin beta-1
β-TCP	Beta-tricalcium phosphate

Gene symbols are *italicized* in the text according to the guidelines of the Human Genome Organization nomenclature committee (HGNC). The gene names used in this book can be found at: http://www.genenames.org/.

1 INTRODUCTION

Bone represents the most transplanted human tissue in Europe with an approximately one million annual procedures. The estimated costs for treating non-healing bone defects are 10 000-100 000 € per patient (Roffi et al. 2017, Stanovici et al. 2016). In the USA, treating bone defects has been estimated to cost at least 5 billion $ per year (Perez et al. 2018). Large bone defects are a major burden on public health which requires the development of effective and feasible new treatment approaches. Currently, autologous bone tissue transfer represents the reference standard with the most predictable outcomes (Hurvitz et al. 2006, Wilkman et al. 2017). However, this is associated with an inherent donor-site morbidity and relative availability-dependence on patient age and general condition. Alternative reconstructive options, which include allogeneic and xenogeneic bone grafts, are not as predictable for sizable defect reconstructions and in compromised recipient-sites (Järvinen et al. 2019, Pogrel et al. 1997).

Bone tissue engineering (BTE) has been expected to achieve a paradigm shift in the reconstructive approaches (Chanchareonsook et al. 2014, Wang et al. 2011). However, despite the significant progress in the *in vitro* and preclinical studies, BTE is largely considered to be over-promised and under-delivered from a clinical perspective to date (Mastrullo et al. 2020, Nerem 2006). The clinical translation for BTE faces critical hurdles related to e.g., conceptual difficulties for the implicated role of employed cells and biomaterials, lack of mature vasculature in large constructs, access to good manufacture practice (GMP) facilities and appropriate regulatory licenses, to name a few (Mastrullo et al. 2020, Williams 2019).

The fundamental elements of BTE approaches involve the cells, e.g., mesenchymal stem/stromal cells (MSCs), biomaterials as an extracellular matrix equivalent, bioactive signals, and the *in vitro/in vivo* regenerative environment. Currently, the exact mechanisms of the biological role of transplanted cells are not fully elucidated. However, realization of the significant paracrine effects of MSCs via their secretome has evolved. Extracellular vesicles (EVs) have emerged as major contributors to intercellular communication. Therefore, research efforts are currently devoted for optimizing the isolation and characterization of EVs as clinically promising biologics (Marolt Presen et al. 2019).

The application of growth factors is an interesting approach from a clinical perspective; however, they are unstable and expensive. Local delivery of vascular endothelial growth factor (VEGF) is promising for enhancing vascularization. Nevertheless, the short half-life reduces its efficacy, rising the dose to super-physiological levels increases potential systemic side-effects and risk of complications (Simón-Yarza et al. 2012). Currently, the use of small molecules, such as prolyl hydroxylase inhibitors (PHIs), is gaining attention as more stable and affordable potential alternative bioactive agents. PHIs could target the activation of hypoxia-inducible transcription factor (HIF-1)/VEGF pathway in MSCs and potentially

enhance their proangiogenic effects. Other biological effects, however, need to be assessed.

One of the clinically promising strategies for translational BTE application is the *in vivo* bioreactor (IVB) strategy. This aims at harnessing the patient body for generating new, customized, and prevascularized autologous tissues for reconstructive applications. This approach combines the potential of conventional reconstructive surgery and BTE (Huang, Kobayashi, et al. 2016, Tan et al. 2004). It is essential, however, to assess the regenerative potential of different IVB techniques in a clinically relevant model with minimal or no exogenous factors or transplanted cells (Huang, Kobayashi, et al. 2016, Huang, Liu, et al. 2016).

The deeper understanding of bone biology, related tissues physiology, and response to injury, together with the advances in biomaterial research could instigate innovative regeneration-driven approaches in the near future (Lopes et al. 2018).

2 REVIEW OF THE LITERATURE

2.1 Bone organ/tissue

The bone is a dynamic organ which constitutes a central component of the musculoskeletal, hematopoietic, and immune systems, additionally, it serves secondary endocrine functions (Brotto and Bonewald 2015). Such dynamic nature of bone, as an organ, is reflected on the structure and interactions of its component tissues: bone/osseous tissue, bone marrow, vascular, epithelial, nerve, and cartilage tissues (Brotto and Bonewald 2015, Kenkre and Bassett 2018).

Osseous tissue endows the mechanical rigidity and load-bearing strength to bone, and is responsible for its locomotor, protective, supportive, and mineral homeostasis functions. Being a specialized connective tissue, bone tissue comprises bone cells and the mineralized extracellular matrix which dictates its biochemical and physical properties (Le et al. 2017).

Behind the inert appearance of bone, there is a continuous dynamic process of bone remodeling which replaces old bone by new bone. Such dynamic process involves the balance and reciprocal control between the bone-forming axis and bone-resorbing arm (Florencio-Silva et al. 2015, Le et al. 2017).

2.2 Bone remodeling

Bone remodeling process is responsible for the maintenance and repair of the skeleton, adaptation to mechanical stresses, and mineral homeostasis. This continuous process replaces old and damaged bone with new, and is tightly regulated within temporary anatomical structures which are the Basic Multicellular Units (BMU) (Frost 1964, Kenkre and Bassett 2018). The balanced bone remodeling is a key determinant of bone strength; microfractures can accumulate with a low bone remodeling, in contrast, excessive resorption causes microarchitectural deterioration of bone (Clarke 2008).

The BMU comprises osteoclasts, osteoblasts, capillary blood vessels, and the peripheral nerve fibers. Osteocytes regulate the continuous replenishment of the BMU with cells, because the BMU's life span is longer than the involved osteoblasts and osteoclasts. The BMU in trabecular bone is housed on the surface where Howship's lacunae are resorbed and subsequently refilled with new bone. In cortical bone, BMUs form cutting cones which burrow tunnels into the remodeling bone to remove the old bone and allow for concentric new bone formation on the tunnel wall around the newly formed vascularized Haversian canal (Kenkre and Bassett 2018). In the BMU, a canopy of cells encapsulates the functioning cells within the bone remodeling compartment (BRC) in a well-reserved specific microenvironment for a close anatomical coupling of osteoclasts and osteoblasts. The BRC formation involves the separation of BLCs from the underlying bone surface, a process orchestrated by the osteocytes (Florencio-Silva et al. 2015, Rochefort et al. 2010).

Currently, the bone-resident macrophage cell (osteomac) has gained attention as an indispensable contributor for bone formation. Osteomacs are attracted to injury and remodeling sites where they contribute to the formation of the cellular canopy of the BRC (Figure 2.1). Within the BRC, a close interaction occurs between the bone cells, endothelial cells, osteomacs, and immune cells. The remodeling cycle involves five overlapping steps of activation, resorption, reversal, formation, and termination (Figure 2.1) (Batoon et al. 2017, Kenkre and Bassett 2018, Le et al. 2017).

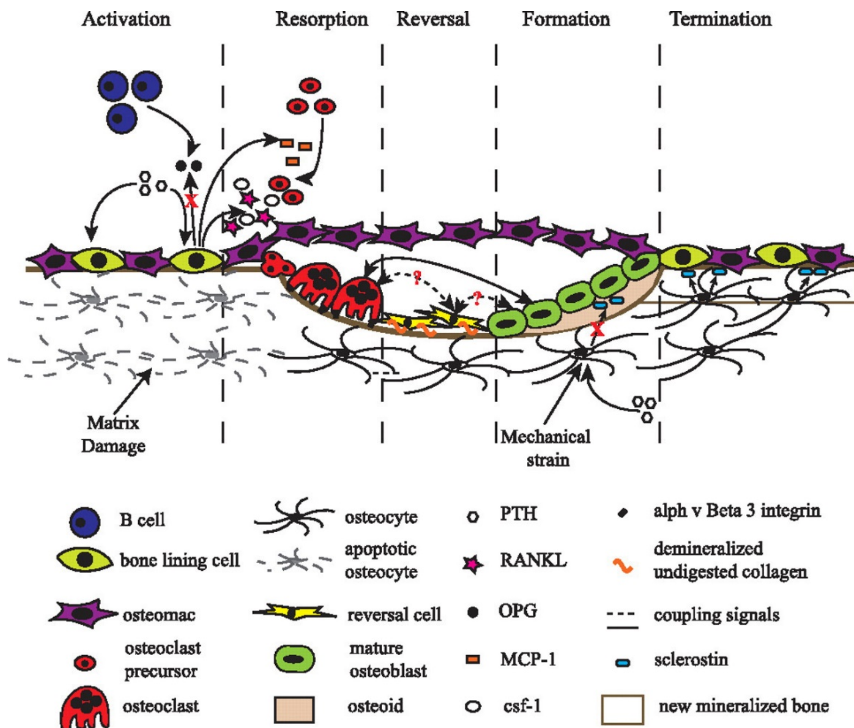

Figure 2.1: Schematic representation of a BMU and bone-remodeling cycle. The resting bone surface is covered with bone-lining cells, preosteoblasts and osteomacs. B-cells, in the bone marrow, secrete OPG which suppresses osteoclastogenesis. During activation, the bone-remodeling signal PTH binds to its receptor on preosteoblasts. Damaged bone ECM results in osteocyte apoptosis, decreasing the local transforming growth factor- β (TGF-β) and its inhibition of osteoclastogenesis. In resorption phase, monocyte chemoattractant protein-1 (MCP-1) is released from osteoblasts and recruits preosteoclasts to the bone surface. Osteoblast expression of OPG is decreased, and production of colony stimulating factor-1 (CSF-1) and RANKL is increased to promote osteoclastic differentiation. Mature osteoclasts anchor to RGD-binding sites to create sealed zone for facilitating degradation of the mineralized bone matrix. During reversal, reversal cells remove demineralized undigested collagen from the bone surface. Transition signals are generated to stop bone resorption and induce bone formation. PTH and mechanical activation of osteocytes reduce sclerostin expression which allows Wnt-directed bone formation to occur. In termination, sclerostin expression returns, and bone formation ceases, new osteoid is mineralized, bone surfaces return to a resting state, and the remodeling cycle concludes. Reproduced from (Raggatt and Partridge 2010) article under CC BY-NC-ND 4.0 license.

2.3 Bone regeneration

Bone has a characteristic regenerative potential for healing injuries without a scar tissue formation. This unique regeneration recapitulates the fetal bone developmental mechanisms: intramembranous and endochondral ossification. Both mechanisms eventually synthesize the same bone tissue, therefore, intramembranous and endochondral terms refer to the replaced developmental tissues (Lopes et al. 2018, Shapiro 2008). In intramembranous bone formation, the inner (cambium) osteogenic layer of the periosteum mediates new bone synthesis without an intermediate cartilage phase. Similarly, in intramembranous bone healing, MSCs directly differentiate along the osteoblast lineage. In contrast, endochondral bone formation involves the synthesis of bone on a predeveloped mineralized-cartilage scaffold (Shapiro 2008).

Craniomaxillofacial bones are developed largely by intramembranous bone formation. Endochondral ossification, however, has been described for the growing mandibular neck/condyle, base of the skull, and temporal and occipital bones (Kruijt Spanjer et al. 2017, Nanci 2017). In both instances, the process starts with a condensation of a cluster (nidus) of undifferentiated MSCs. Craniomaxillofacial MSCs uniquely originate from two robust mesenchymal populations: the ectomesenchymal neural crest cells (NCCs) and the paraxial mesoderm (Hall 2008, Kruijt Spanjer et al. 2017, Noden and Trainor 2005).

2.3.1 Mechanisms of clinical bone healing

In the clinical bone injury situations, e.g., fractures, osteotomies, and bone defects, the optimal healing should completely reconstitute the bone across the injury site. The healed bone should be remodeled and seamlessly incorporated into the adjacent bone. Bone healing mechanisms largely depend on the provided biomechanical environment, as up to 10% of bone fractures do not heal predictably (Einhorn and Gerstenfeld 2015, Shapiro 2008).

Primary bone healing occurs in an environment of rigid fixation and direct interfragmentary contact. Such direct contact allows the crossing of BMU cutting cones from either fracture side to resorb necrotic bone and directly synthesize remodeled lamellar bone. If an interfragmentary space of more than 0.1 mm exists with a rigid fixation, direct (transformational) bone healing will occur. Blood vessels and MSCs mediates the direct healing without a cartilage phase, as MSCs home into the interfragmentary gap and differentiate into osteoblasts to synthesize randomly oriented woven bone which is remodeled later into lamellar bone. Secondary (endochondral) bone repair occurs by callus formation, when an interfragmentary space and/or non-rigid fixation exists. The cartilaginous callus undergoes endochondral ossification to form woven bone which is remodeled into lamellar bone (Einhorn and Gerstenfeld 2015, Shapiro 2008).

2.3.2 Vascularization and bone healing

Bone healing mechanisms share a common crucial feature, which is prevascularization, for regenerating fully functional bone. In endochondral ossification, clustered MSCs differentiate into chondrocytes via the activation of SRY (sex determining region Y)-box 9 (Sox9) and the suppression of β-catenin. Under the control of transcriptional factor Sox9, chondrocytes proliferate before undergoing hypertrophy through Runt-related transcription factor 2 (Runx2) activation which is further regulated by oxygen tension. The hypertrophic chondrocytes within the secreted cartilaginous matrix undergo apoptosis, eliciting a cascade of paracrine signals to recruit endothelial cells and angiogenesis (García and García 2016, Mackie et al. 2008). The critical regulators for those steps include hypoxia-inducible factor-1 (HIF-1α) and vascular endothelial growth factor (VEGF) (Lopes et al. 2018).

HIF-1α mediates the hypoxic responses which are critical for the survival of hypoxic chondrocytes. In addition to its osteocytes survival role; VEGF, which is one of the HIF-1α targets, plays a critical role in the vascular invasion of the cartilaginous matrix under the control of Runx2 (Mackie et al. 2008, Schipani 2005, Zelzer et al. 2001). The vascular invasion is followed by the osteoclastic activity on the cartilaginous matrix in order to condition it for the orderly invasion for the ossification front (García and García 2016, Mackie et al. 2008, Schipani 2005).

In contrast to the well-established critical role of angiogenesis during endochondral ossification, the evidence for the importance of angiogenesis during intramembranous ossification is cumulating (Percival and Richtsmeier 2013). Like endochondral ossification, it is generally assumed that hypoxia of the avascular mesenchymal condensations is a critical regulator for the angiogenesis-osteogenesis coupling during intramembranous ossification. However, angiogenesis regulatory pathways in the different osteogenesis mechanisms are distinct with different expression patterns of the HIF-alpha subunits (De Spiegelaere et al. 2010, Percival and Richtsmeier 2013). Distraction osteogenesis (DO), a standard technique for bone lengthening, represents a valid intramembranous ossification model for studying angiogenesis-osteogenesis coupling. Vascular ingrowth from the medullary sinusoids of the distracted edges and periosteal vessels form new vascular sinuses, around which new bone formation starts by their accompanying osteoblasts along the tense collagen bundles (Choi et al. 2002, Percival and Richtsmeier 2013). Treatment with an angiogenic inhibitor in DO rat model halted both bone regeneration and blood vessel formation. Therefore, it is evident that both the mechanical environment and angiogenesis are determinants for intramembranous osteogenesis (Fang et al. 2005, Percival and Richtsmeier 2013). It is hypothesized that angiogenesis critically spatio-temporally precedes the appearance of osteoblasts and mineralized bone. Such critical regulation involves the interplay of currently unidentified anti-angiogenic factors and other known proangiogenic factors; i.e., VEGF and HIF (Percival and Richtsmeier 2013).

2.3.3 Periosteum-Sharpey's fiber-endosteum system

The periosteum is a specialized fibrous connective tissue sheath which covers the non-articular cortical surface of bone to which it is tightly attached by thick collagenous Sharpey's fibers. The periosteum consists of an outer fibrous layer and inner osteogenic *cambium* layer (Figure 2.2). The endosteum is a connective tissue membrane which lines the inner surfaces of bone and its vascular Volkmann's canals (Figure 2.2). Both periosteum and endosteum are well-vascularized and innervated structures and rich sources of bone cells (Clarke 2008).

Sharpey's fibers, i.e., perforating fibers, enter the outer circumferential and interstitial lamellae of bone tissue and are particularly abundant in the dental alveolar sockets. Sharpey's fibers were first described in the seventeenth century by Clopton Havers as penetrating periosteal "fibrillae", however, they were later named after William Sharpey in the nineteenth century (Aaron and Skerry 1994, Dobson 1952). Concurrent with their later description, H. Muller reported on the elastic nature of Sharpey's fibers and their tendency to evade calcification (Aaron 2012).

Aaron and Skerry studied the regeneration of bone trabeculae after a localized ablation in an adult sheep iliac crest in comparison to the normal intramembranous trabecular formation in a fetal lamb, common striking features were evident. Both the damaged endosteum in sheep and the intact periosteum in lambs similarly produced arrays of distinct course collagenous fibers. Those migrating arrays of fibers penetrated the soft tissues to form a preliminary polarized framework, which was a precondition for developing bony trabeculae. The preliminary framework served to bond soft to hard tissues and old to new bone (Aaron and Skerry 1994). Periosteal/endosteal Sharpey's fibers aim to reestablish the lost continuity of the injured site by picking up the damaged threads and bony fragments, connecting them to the excised bony surfaces for scaffolding trabecular intramembranous ossification. It is proposed that a periosteum-Sharpey's fibers-endosteum system exists, not only as a structural continuum, but also as an important bone matrix regulatory system (Aaron 2012).

2.3.4 The *critical* critical-size bone defect

Given the unique regenerative capacity of bone, most bone defects can heal spontaneously under a balanced biological and mechanical microenvironment (Ihan Hren and Miljavec 2008, Reichert et al. 2009). Nevertheless, an aversive soft tissue environment, large bone defects, or biomechanical instability can limit the intrinsic regeneration potential and create non-healing bony defects (Reichert et al. 2009). This should not be confused with fracture nonunion, which often occurs without a bone gap, due to, e.g., impaired biological conditions. Large bone defects associated with inability to replace substantial bone loss despite the adequate biology are described as the so-called critical-size defects (Schemitsch 2017).

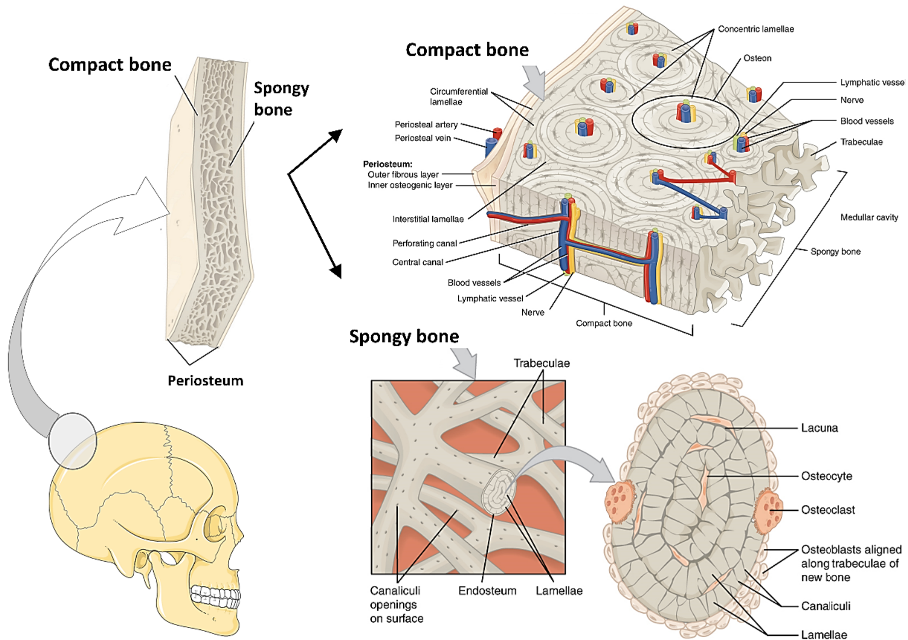

Figure 2.2: Basic structure of lamellar bone. Adapted and combined from (Servier-Medical-Art) and (OpenStax-College) from Wikimedia Commons; "606 Spongy Bone"; "621 Anatomy of a Flat Bone"; "624 Diagram of Compact Bone-new", modified, https://creativecommons.org/licenses/by/3.0/legalcode

The concept of the critical-size defect (CSD) was introduced in the experimental setting by Schmitz and Hollinger as the defect of *a size* that will not heal during the lifetime of the animal (Schmitz and Hollinger 1986). For practical experimental reasons, a CSD is defined by *the minimum defect size* which does not exceed 10% of spontaneous bone regeneration during the experimental time course (McGovern et al. 2018).

Despite the initial focus on the size factor of the CSD, it was also appreciated that it should be particularly specified for each bone. The CSD has been proposed, in the standard experimental guidelines, to be a segmental continuity defect of a length exceeding 2.5 times the diameter of the affected bone (ASTM 2014). Nevertheless, in a canine femur model, a defect of approximately 2 times the mean diameter of the femur diaphysis proved to be a CSD (Lindsey et al. 2006). In sheep tibiae, however, the CSD was 3 times the corresponding diameter (Gugala and Gogolewski 1999, Gugala et al. 2007).

Interestingly, Lammens and coworkers reported a *warning* over the sheep tibial CSD model. They documented that even a 4.5 cm defect, which is larger than the *so-called* CSD, in young (immature) sheep showed a partial regeneration in up to 25% of the animals despite the excision of the periosteum. Consequently, the authors suggested

paying *a critical* attention to other factors, e.g., maturation status, presence of the periosteum, and the adequate fixation (Lammens et al. 2017).

It is an ongoing challenge to establish the optimum preclinical model for bone defect research. Defining the CSD only by its size is insufficient; CSD is additionally a product of the species, bone metabolic/remodeling rate, anatomic location, presence of periosteum, soft tissue envelope, and mechanical stresses. Additionally, it is further complicated, especially in the clinical setting, by the host's age, nutrition, metabolic and systemic conditions, and related morbidities (Ho-Shui-Ling et al. 2018, Lindsey et al. 2006, McGovern et al. 2018, Reichert et al. 2009). Therefore, caution should be exerted while translating preclinical studies into human clinical scenarios which are usually complicated, for example, by poor soft tissue conditions or infection. In such cases, even a defect smaller than the assumed CSD limit would not heal due to other associated factors (Schemitsch 2017).

A preclinical animal model should fulfill specific requirements regarding its anatomy and size. Evaluation of orthopedic/prosthetic devices involves a large-sized non-human primates' model, e.g., dogs, pigs, sheep, and goats (Martini et al. 2001). Dogs have been considered as the closest model to humans regarding their bone weight, density, and bone material. Nevertheless, dogs and humans are different in their remodeling rate and bone microstructure. Currently, ethical issues have complicated the use of dogs in orthopedic and trauma research (Aerssens et al. 1998, Martini et al. 2001, Reichert et al. 2009). Despite the differences in the bone histology between sheep and humans, e.g., density of Haversian canals, the bone healing rate in sheep is approximately similar to humans. Additionally, sheep anatomy allows the use of plates/implants which are designed for use in humans (Den Boer et al. 1999). Pigs are highly representative model for human bone regeneration, due to their healing capacity, remodeling rate, and bone mineral density. Pigs are, however, relatively difficult to handle compared with sheep, and the human stock-implants are frequently incompatible for application in pigs (Aerssens et al. 1998, Reichert et al. 2009).

2.3.5 Osteoconduction, osteoinduction, and osteogenesis

For the management of bone defects, certain principal terms usually describe the behavior of different grafts/materials in the context of bone regeneration. *Osteoconductive* materials can guide the bone-forming tissue from the periphery of a given bone defect. *Osteoinduction* is the ability to induce bone formation by attracting and stimulating bone-forming host cells at the recipient site which could be an orthotopic skeletal site or even in an appropriate heterotopic site. *Osteogenesis* is defined as the capability to form bone tissue *de novo*, which implies the activity of osteoblastic cells within the osteogenic grafts/constructs independent on the homing of the host cells (Cooper et al. 2001, Cornell and Lane 1998, Gotoh et al. 1995, Hotz and Herr 1994, Kneser et al. 2002).

2.4 Bone cells

Bone cells are generally categorized under two lineages: the bone-forming osteoblast lineage and the bone resorbing osteoclast lineage. The osteoblast lineage originates from mesenchymal stem/stromal cells (MSCs) and comprises preosteoblasts, mature osteoblasts, bone lining cells, and osteocytes. The bone marrow hematopoietic stem cells differentiate into osteoclast lineage cells, which include macrophages, osteoclasts, and multinucleated giant cells (Le et al. 2017).

2.4.1 MSCs, preosteoblasts, and osteoblasts

Osteoblasts are responsible of building bone tissue; they secrete osteoid bone matrix proteins and guide its subsequent mineralization. The short-lived mature osteoblasts are continuously supplemented by preosteoblasts and MSCs. While maintaining their stock by self-renewal capacity, undifferentiated MSCs reside in the perivascular, bone marrow, endosteum, and periosteum compartments bounding differentiated cells (Florencio-Silva et al. 2015, Le et al. 2017).

Multipotent MSCs are the precursors of osteoblasts and chondrocytes, thus playing a critical role in both bone healing and remodeling processes. Currently, more understanding is evolving for the role of MSCs in modulating and establishing their regenerative microenvironment. Such role involves MSCs-secreted products, thus appreciating MSCs as an *in vivo drugstore* (Caplan and Correa 2011, Le et al. 2017).

Much of our understanding for MSCs therapeutic and regenerative potential is based on *in vitro* studies, paradoxically, MSCs are one of the most employed regenerative cells in clinical trials despite the existing questions regarding their origin, native identity, and *in vivo* biology (Da Silva Meirelles et al. 2008, Le et al. 2017, Murray and Péault 2015).

In vivo commitment of MSCs to osteoblast lineage involves critical synchronization of programmed steps which are not fully elucidated. *In vitro* osteogenic differentiation of MSCs and experiments involving genetically modified mouse models highlighted some of the master events. Early events involve bone morphogenetic proteins (BMPs) and Wingless (Wnt) family members pathways for promoting MSCs commitment towards osteoblastic lineage (Figure 2.3). Osteoprogenitor cells express Runx2, Distal-less homeobox 5 (Dlx5), and Osterix (Osx) (Capulli et al. 2014).

Runx2 is a master gene for osteoblastic differentiation, genetically modified mice with global Runx2 deletion showed a complete lack of mature osteoblasts and failure of bone formation. Interestingly, Runx2 has a pivotal role during the early stages of osteogenesis and osteoblastic differentiation, which is not equally crucial for already committed osteoblasts (Komori et al. 1997, Takarada et al. 2013). *Runx2* upregulates type I collagen alpha 1 *(CollA1)*, alkaline phosphatase *(ALPL)*, bone sialoprotein *(BSP)*,

and osteocalcin *(BGLAP)*, all are osteoblast-related genes (Fakhry et al. 2013, Florencio-Silva et al. 2015).

Osx, also known as Sp7, is another transcriptional factor which is critical for osteoblastic differentiation (Karsenty 2008). Osx is a downstream target of Runx2, as it is not expressed in *Runx2*-defecient mice. *Osx*-defecient mice, however, express Runx2, while lacking normal bone formation. Such effect was more evident in the bones formed by intramembranous ossification as compared to those formed by endochondral ossification. Therefore, Osx is crucial for directing MSCs towards osteoprogenitors during bone formation *in vivo* (Karsenty 2008, Nakashima et al. 2002).

Dlx5 is an activator for *Runx2* and is considered as a positive regulator of osteoblastic differentiation. *Dlx5*-null mouse embryos showed decreased bone volume of their femurs due to deficient osteoblastic activity, which was associated with decreased *Runx2* expression (Samee et al. 2008). *Dlx5* can, however, promote osteoblast-related genes independently of *Runx2* (Hassan et al. 2006). Similarly, *Osx* can be activated by Dlx5; indirectly via *Runx2* activation, or directly in a Runx2-independent mechanism (Lee et al. 2003, Samee et al. 2008). During osteoblastic differentiation, Dlx5 plays a master regulatory role with other transcriptional factors, however, it is not as crucial as Runx2 and Osx (Heo et al. 2017, Samee et al. 2008).

At a post-transcriptional level, micro RNAs (miRNAs) came into the scene as important regulators for gene expression during osteoblastic differentiation. The miRNAs are small (19-25 nt) single stranded endogenous non protein encoding RNAs, which can exert regulatory functions via targeting mRNAs. Their effect on osteoblastic differentiation could be either positive or negative depending on the targeted mRNAs (Capulli et al. 2014). Several miRNAs have been demonstrated to impede osteoblastic differentiation by targeting Runx2, e.g., miR-23a, miR-30c, miR-34c, miR-133a, miR-135a, miR-137, miR-204, miR-205, miR-217, and miR-338 (Ying Zhang et al. 2011). Osx can be specifically inhibited by miR-637 (J. F. Zhang et al. 2011). In contrast, other miRNAs can promote osteoblastic differentiation, including miR-21, miR-217, miR-26a, miR-148a, miR-200b, miR-335-5p, miR-92a, miR-9, and miR-199b-5p (Jicheng Wang et al. 2019). The targeted pathways for pro-osteoblastic differentiation include, for example, GSK-3β/β-catenin signaling pathway (suppressed by miR-199b-5p) and PTEN/PI3K/Akt/HIF-1α pathway (activated by miRNA-21) (Jicheng Wang et al. 2019, Yang et al. 2019, Zhao et al. 2016).

Osteoprogenitors, expressing Runx2 and collagen I, enter a proliferative phase with a greatly-reduced plasticity (Blair et al. 2008). They acquire alkaline phosphatase activity (ALP), which characterizes their transition into preosteoblasts (Capulli et al. 2014). Preosteoblasts become more actively secreting bone matrix proteins, with higher ALP activity. Moreover, they undergo morphological changes, becoming large, cuboidal cells while developing into mature osteoblasts (Capulli et al. 2014, Florencio-Silva et al. 2015).

Mature osteoblasts stop proliferating and show an increased expression of Osx and active secretion of bone matrix proteins: osteocalcin (OCN), bone sialoprotein (BSP) I/II, and collagen type I. Osteoblasts synthesize organic bone matrix, or osteoid, in which they will be eventually trapped; giving rise to the osteocytes (Figure 2.3). The aging osteoblast can either undergo apoptosis or become a bone lining cell (Capulli et al. 2014).

2.4.2 Bone lining cells

Bone lining cells (BLCs) are flattened quiescent osteoblasts with a non-fully understood function. Despite the lack of effective techniques for selective isolation and characterization of BLCs, they can be distinguished from osteoblasts by being positive for intercellular adhesion molecule 1 (ICAM1) (Everts et al. 2002). BLCs cover the bone surfaces where there is no active bone formation/resorption, nevertheless, their cytoplasmic organelles and gap junctions with adjacent BLCs/osteocytes indicate an active homeostatic role (Florencio-Silva et al. 2015, Miller et al. 1989).

BLCs are considered as determined osteogenic precursors; depending on the physiological status of bone, these cells can reacquire an active secretory phenotype and act as a major source of osteoblasts during adulthood (Matic et al. 2016). BLCs can play a protective role against bone resorption by preventing the direct interaction between osteoclasts and bone matrix. BLCs participate, however, in the osteoclastic differentiation by producing osteoprotegerin (OPG) and the receptor activator of nuclear factor kappa-B ligand (RANKL). Therefore, BLCs are an important element of the basic multicellular unit (BMU) during the process of bone remodeling (Andersen et al. 2009, Everts et al. 2002, Florencio-Silva et al. 2015, Kenkre and Bassett 2018).

2.4.3 Osteocytes

Osteocytes are the most abundant cells in the mammalian bone tissue, these long-lived cells are estimated to constitute 95% of bone cellular content and cover 94% of bone surfaces with an average half-life of up to 25 years (Franz-Odendaal et al. 2006, Frost 1960, Marotti 1996). Osteocytes have been described, since more than 150 years, to originate from osteoblasts (Gegenbaur 1864). An osteoblast gets progressively entrapped within the mineralizing bone matrix, thus developing the lacuna which houses the osteocyte cell body. During the osteoblast-osteocyte transition, cells develop dendritic processes (up to 50 long and branched processes), downregulate osteoblastic markers, and highly express osteocyte markers including dentine matrix protein 1 (*DMP1*) and sclerostin (*SOST*) (Figure 2.3) (Bonewald 2011).

Osteocytic dendrites extend into channels or *canaliculi* in the matrix to communicate with each other and with other cells on the bone surface. Such lacuno-canalicular system creates an extensive molecular exchange surface area (Rochefort et al. 2010). In contrast to the old erroneous notion of the passivity of the osteocytes, these cells have been recognized to exert several important functions (Bonewald 2011).

The characteristic tridimensional osteocyte syncytium works as a mechano-transduction system within the bone matrix. Osteocytes can detect mechanical loads; directly through the solid matrix or indirectly via the interstitial fluid pressure in the lacuno-canalicular system. Osteocytes, through their piezoelectric effect, translate the mechanical stimuli into biochemical signals which regulate osteoblastic and osteoclastic activities. Therefore, osteocytes orchestrate bone remodeling and adaptation to daily mechanical stresses (Capulli et al. 2014, Florencio-Silva et al. 2015, Knothe Tate 2003). Additionally, osteocytes have an important role in mineral homeostasis, and phosphate metabolism among other secondary endocrine functions (Capulli et al. 2014, Martin et al. 2011).

The very special location of the osteocytes and their functions have inspired the literature with some astonishing metaphors, e.g., "choreography from the tomb", "buried alive", and "martyrs for the integrity of bone strength", reflecting the role played by osteocytes apoptosis on bone remodeling regulation (Franz-Odendaal et al. 2006, Manolagas 2006, Rochefort et al. 2010, Seeman 2006). Thus, bone cells constitute a functional continuum from preosteoblasts to mature osteocytes, within which cells at all stages of bone formation remain connected (Franz-Odendaal et al. 2006).

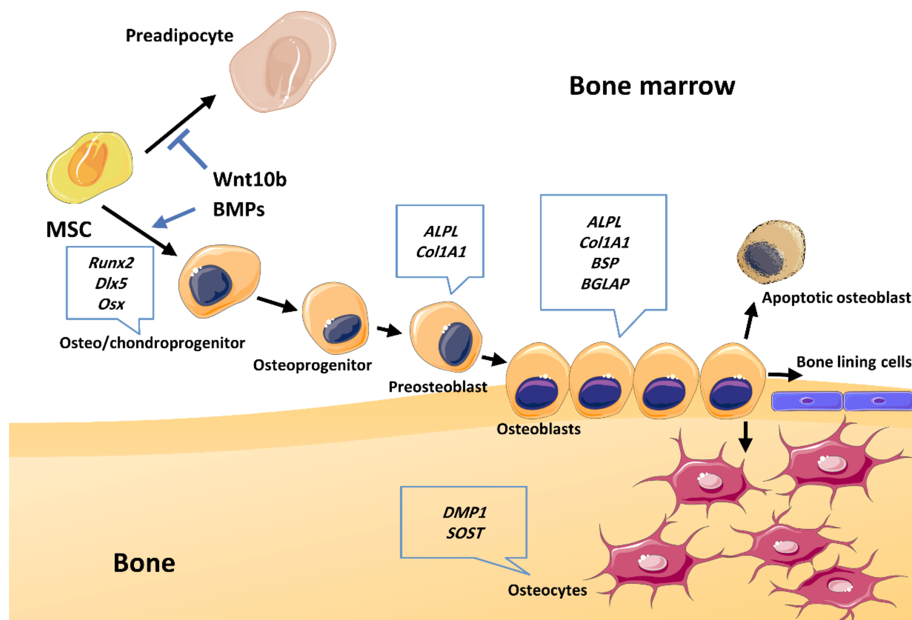

Figure 2.3: Schematic representation for osteoblastic differentiation and fate. The early events which promote the commitment of multipotent mesenchymal stem/stromal cells (MSC) towards osteoblastic lineage and the fate of mature osteoblasts are depicted. Adapted with permission from (Capulli et al. 2014) and created using images from (Servier-Medical-Art).

2.4.4 Osteoclasts

Osteoclasts are terminally differentiated multinucleated cells which originate from the fusion of mononuclear cells of the monocyte/macrophage family from the hematopoietic stem cell lineage. Osteoclasts are uniquely able to resorb mineralized bone tissue. Several factors are involved in activating osteoclastogenesis (Figure 2.4), the most important of those factors; the macrophage colony-stimulating factor (M-CSF) and RANKL, are produced by osteoprogenitor cells, osteoblasts, and osteocytes (Florencio-Silva et al. 2015, Han et al. 2018, Plotkin et al. 2019).

Osteoclasts can originate from both immature cells of the monocyte-macrophage lineage as well as mature tissue macrophages in a suitable microenvironment created by osteoblast lineage cells. Additionally, the contact between osteoclast precursors and osteoblast lineage cells is essential for osteoclastogenesis (Teitelbaum 2000, Udagawa et al. 1990). M-CSF is a secreted product from MSCs and osteoblasts which is essential for macrophage maturation, it binds to c-Fms receptor on osteoclast early precursors and promotes their survival and proliferation (Figure 2.4) (Teitelbaum 2000, Udagawa et al. 1990). The necessity of cell-to-cell contact between osteoclast precursors and osteoblast lineage cells, together with the presence of receptor activator of nuclear factor kappa-B (RANK) on osteoclasts and their precursors, indicated that RANKL resides on osteoblast lineage cells and acts as an osteoclast-differentiating factor. Osteoprotegerin (OPG) is largely expressed by osteoblast lineage cells (Figure 2.4), and acts as a soluble decoy receptor which competes with RANK for RANKL (Lacey et al. 1998, Teitelbaum 2000).

The balance between bone resorption and formation involves the RANKL/RANK/OPG system, which mediates the interaction between osteoclasts and osteoblast lineage cells (Florencio-Silva et al. 2015). Osteoblast lineage cells are the targets for most osteoclastogenic agents, e.g., parathyroid hormone (PTH), which act through enhancing RANKL and decreasing OPG expression (Teitelbaum 2000). Additionally, osteoclasts proved to be capable of modulating osteoblastic new bone formation, osteoclasts release extracellular vesicles (EVs) which carry RANK on their surface (Figure 2.4), vesicular RANK binds to RANKL on the osteoblasts directing them to form new bone (Ikebuchi et al. 2018).

2.5 Bone extracellular matrix (ECM)

Osseous interstitial ECM has a composite structure consisting of an organic matrix (collagens, non-collagenous proteins, proteoglycans, and glycosaminoglycans) which binds tightly to hydroxyapatite (the mineral component) (Le et al. 2017). Over 30% of the acellular part of bone tissue consists of organic components, 90% of which is collagenous proteins, predominantly type I collagen (Florencio-Silva et al. 2015). The organization of collagen and its physical properties with its subsequent mineralization is a key determinant for the mechanical strength of bone (Viguet-Carrin et al. 2006). Additionally, ECM proteins bind to several bioactive and adhesion

molecules, e.g., integrins and RGD, which provide a unique interface for interaction with bone cells (Florencio-Silva et al. 2015, Le et al. 2017).

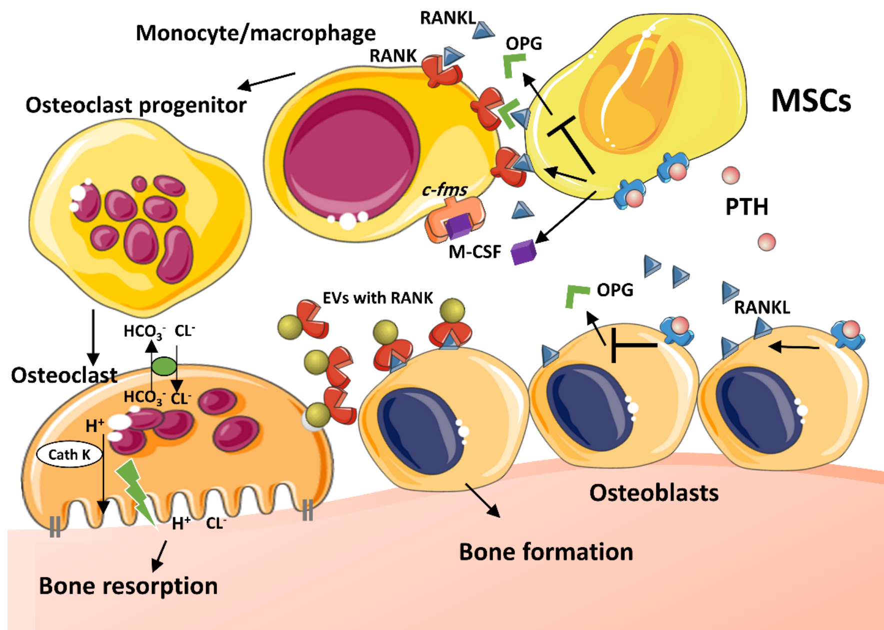

Figure 2.4: Schematic illustration for the osteoclastogenesis, osteoclastic bone resorption, and the reciprocal interactions between osteoblasts and osteoclasts. Osteoprogenitors and osteoblasts express RANKL and M-CSF when stimulated by osteoclastogenic molecules, e.g., PTH, which also reduce OPG expression. RANKL and M-CSF bind their receptors on monocyte/macrophage cells and induce commitment to the osteoclast phenotype, this process is inhibited by OPG. The mature osteoclast polarizes on the bone surface and creates the bone resorptive acidic microenvironment. Osteoclasts also modulate osteoblastic activity through the release of extracellular vesicles (EVs) that contain RANK on their surface. Adapted with permission from AAAS (Teitelbaum 2000) and Elsevier (Lopes et al. 2018), created using images from (Servier-Medical-Art).

The inorganic mineral component of bone is blended into the living matrix of cells and proteins, forming the bone composite. Osteoblasts orchestrate the mineralization process by interacting with the osteoid tissue via releasing matrix vesicles, where calcium ions are immobilized and nucleate with the ALP-released phosphate ions to form the carbonate-substituted hydroxyapatite crystals ($Ca_5(PO_4)_3OH$). The supersaturated matrix vesicles rupture and the crystals spread into the surrounding matrix in tandem with the arrangement of collagen fibrils (Florencio-Silva et al. 2015, Le et al. 2017).

2.6 Craniomaxillofacial bone defects

2.6.1 Etiology and incidence

Surgeons are confronted by craniomaxillofacial bone defects on a weekly basis (Kruijt Spanjer et al. 2017). Such defects stem from trauma, tumor ablation, congenital defects, and bone pathological conditions. Craniomaxillofacial trauma can lead to disabling injuries which may require complex reconstructive treatment especially after a high-energy trauma. A recent Global Burden of Disease Study (GBD) has estimated the new cases of facial fractures, in 2017, to be approximately 7.5 million cases, with 1.8 million individuals suffering disability due to a facial fracture (Lalloo et al. 2020).

Falls are the global leading cause of craniomaxillofacial injuries, however, in conflict regions, e.g., North Africa and Middle East, high-energy mechanism injuries are common (Lalloo et al. 2020). Such injuries affect both the military and the civilian populations, which emphasizes the need for steady communication between both communities to deal with such complex cases in such complex situations (Farber et al. 2019, Lanigan et al. 2017, Simon et al. 2015).

Resection of tumors in the maxillofacial region often imposes a reconstructive challenge. Given the current global trend for an increased incidence in lip and oral cavity cancers during the last three decades, especially in women and younger age groups, the need for a predictable and long-term stable reconstruction is critical (Du et al. 2019).

Craniofacial anomalies also cause a substantial clinical burden; cleft lip and palate, for example, have a global frequency of 1 per 700 live births (Murray 2002). In addition to the mentioned causes, cranial bone defects could be iatrogenic after neurosurgical or maxillofacial procedures. The cranial defects in particular can predispose for seizures or visual impairment which in turn increase the vulnerability for further traumatic accidents (Li et al. 2021). Craniomaxillofacial bone defects in young patients can affect their normal craniofacial development, therefore, the reconstructive approaches should be designed to restore form and function without further disturbing future craniofacial growth (Piitulainen et al. 2019).

Other causes for craniomaxillofacial bone defects include bone pathologies, e.g., osteomyelitis, osteoradionecrosis, and medication-related osteonecrosis of the jaws (MRONJ). The latter is an emerging complication of the antiresorptive and antiangiogenic therapies in the increasing aging population (Limones et al. 2020). The complex craniomaxillofacial anatomy and adaptation to functions pose a challenging need for perfection in reconstructing CSDs of the craniomaxillofacial skeleton (Kruijt Spanjer et al. 2017).

2.6.2 Reconstructive approaches

The craniomaxillofacial complex is not only necessary for the integrity of vital organs, e.g., brain and eyes, but also vital for mastication, swallowing, upper airway patency, taste sensation, speech, esthetics, and social communication. Craniomaxillofacial soft tissue and/or bony defects can deteriorate those functions and cause a significant deformity, which negatively affects the patient's health, social interaction, self-esteem, psychological well-being, and quality of life (Järvinen et al. 2019, Roumanas et al. 2006, Schliephake et al. 1995).

2.6.2.1 Reconstructive ladder/elevator concept

During the World War I, Harold Gillies; the father of modern plastic surgery, introduced the concept of the *reconstructive ladder* in wound closure. This was after his experience in managing complex maxillofacial wounds while coworking with Charles Valadier and Hippolyte Morestin (McAuley 1974). *Reconstructive ladder* has been popularized as a framework for managing complex wounds (Levin 1993, Mathes and Nahai 1982, Tintle and Levin 2013). Upon climbing the *reconstructive ladder,* the surgeon first encounters simple methods, e.g., allowing the wound to heal by secondary intention, before moving to other more elegant and complex methods, e.g., free tissue transfer (Mardini et al. 2005). Obviously, the core rationale there is to achieve wound coverage in the simplest way with minimal focus on functional outcomes. Consequently, several modifications have been proposed, for example, Gottlieb and Krieger introduced the *reconstructive elevator* concept to suggest a freedom to ascend directly to more advanced methods for achieving better functional outcomes (Gottlieb and Krieger 1994, Janis et al. 2011).

The evolution of predictable microsurgical techniques, over the last sixty years, has achieved a paradigm shift in the reconstructive approaches, not only in plastic surgery, but also in orthopedics (Tintle and Levin 2013). It has been suggested that experts in every reconstructive field should switch their focus from the *ladder/elevator* concept to a new individualized approach which focuses on the time- and cost-efficient restoration for form and function, and improves the patient's quality of life (Al Deek and Wei 2017).

2.6.2.2 The free osseous/composite flaps

For the soft tissue defects, the reconstructive surgeon has a plethora of flap options. These span a wide range of complexity ranging from local and pedicled flaps to free and chimeric flaps. The evolution of the perforator flap concept has even expanded the reconstructive armamentarium with approximately 400 perforators in the body, thus allowing enormous potential for freestyle perforator flap harvest (Kim and Kim 2015, Morris et al. 2010). The functional reconstruction of complex craniomaxillofacial defects requires replacing like with like. Unlike soft tissue flaps, the bone-containing composite flap options are relatively limited, for example, the

free osteocutaneous flaps include a handful of options: fibular, scapular, iliac crest, and osteocutaneous radial forearm flaps (Alrajhi et al. 2013).

Despite the inherent donor-site morbidity and limited availability, free vascularized bone flaps are appreciated as the reference standard. These vascularized flaps can bridge larger bony defects, in both irradiated and non-irradiated fields. Free vascularized bone flaps can be combined with their myocutaneous elements (composite free flaps) or combined with different free flaps to reconstruct extensive bone and soft tissue defects (Hurvitz et al. 2006, Wilkman et al. 2017).

The radial bone offers a 5-12 cm segment of bone in the osteocutaneous radial forearm flap, the bony component, however, may not tolerate multiple osteotomies or insertion of dental implants (Hurvitz et al. 2006). Additionally, the popularity of osteocutaneous radial forearm flap has waned due to the significant incidence of donor-site fractures, which necessitates prophylactic plating of the donor radius (Villaret and Futran 2003, Werle et al. 2000).

The fibular free flap has revolutionized maxillomandibular reconstructions, it features a long segment of robust bone and relatively low donor-site morbidity and allows for a two-team approach. Nevertheless, its soft tissue pedicle is not optimal, and it is not suitable for patients with peripheral vascular disease (Alrajhi et al. 2013, Hidalgo 1989, Hurvitz et al. 2006). Fibular free flaps have been associated with higher rates of osteosynthesis plates related complications, which can affect up to 26% of cases (Dean et al. 2020, Tsang et al. 2017).

In defects with a large soft tissue component and in elderly patients, the scapular osteocutaneous free flap is advantageous. Aside from being challenging for a two-team approach, it has a limited bony stock when considering dental rehabilitation by osseointegrated implants (Alrajhi et al. 2013, Hurvitz et al. 2006, Urken et al. 2001).

The iliac crest flap provides a large stock of soft tissue and robust bone which is essentially curved like a hemimandible/maxilla. Despite these advantages, the iliac crest flap is associated with a considerable donor-site morbidity, bulky soft tissue component, and a short vascular pedicle which limit its application only to carefully selected patients (Cordeiro et al. 1999, Hurvitz et al. 2006). It has been documented that microvascular bone composite flaps undergo resorption for several years, the volume reduction was reported least in the fibula followed by the iliac crest and greatest in scapular flaps (Wilkman et al. 2017).

2.6.2.3 The pedicled osseous flaps

Several regional composite flaps are available for craniomaxillofacial reconstruction. Few examples include the sternocleidomastoid composite flap, latissimus dorsi composite flap, and trapezius composite flap (Chen and Chang 2015, Conley 1972, Wei et al. 2013). Although considered by many surgeons to be outdated, the pedicled osseous flaps remain an excellent reconstructive option. Despite the limited bone

stock, the advantages of pedicled osseous flaps include being technically less-demanding, having lower donor-site morbidity, and providing a reliable alternative in selected cases, e.g., in vessel-depleted necks (Lane et al. 1994, Mahieu et al. 2016, Zaid and Schlieve 2020).

During the current pandemic COVID-19, the reconstructive decision making has been influenced by the need to reduce the operative times, the number of personnel in the operating theater, and the need for postoperative intensive care units. In such critical times, pedicled flaps are appreciated as potential alternatives to free flaps with reliable results in selected cases (Kiong et al. 2020, Mehanna et al. 2020, Rampinelli et al. 2020, Thompson et al. 2020). The limited bony component, however, should be appropriately considered, not to compromise the functional outcomes (Lane et al. 1994, Zaid and Schlieve 2020).

2.6.2.4 Nonvascularized autologous bone grafts

Nonvascularized autologous bone grafts (NABGs) have been employed over the last two centuries in craniomaxillofacial reconstruction (Jackson et al. 1986, McCarthy and Zide 1984). Their use has been popular after the formulation of related surgical and physiological aspects in the early 1960s (Boyne 1969, Burwell 1964, Pogrel et al. 1997). Harvesting NABGs is technically less demanding, it is feasible from multiple donor sites, e.g., iliac crest, ribs, and calvarium. NABGs are the graft of choice for many surgeons, however, high complication rates have been documented. In addition to donor-site morbidity, grafting the mandibular defects with NABGs was associated with 19.7% complication rate at the recipient-site, with a higher complication rate of 25.8% reported for maxillary grafting (Valentini et al. 2007).

NABGs are highly dependent on the recipient-site conditions, they are better reserved for cases with benign conditions and nonirradiated patients. Adequate soft tissue of good quality should be available at the recipient-site to ensure good and tension-free closure (Pogrel et al. 1997). Any conditions affecting the vascularity and viability of the recipient site would jeopardize the graft incorporation. Such conditions could be local, e.g., infection and radiotherapy, or systemic conditions, which affect the normal healing capacity (Elsalanty and Genecov 2009).

In mandibular reconstruction, for example, primary grafting from an intraoral approach is possible, more success was reported, however, using an extraoral approach as a secondary procedure (Obwegeser 1968, Pogrel et al. 1997, Tidstrom and Keller 1990). The failure rate of NABGs is directly proportional to the length/size of the defect, NABGs longer than 6 cm are often associated with higher failure rates (Pogrel et al. 1997).

NABGs undergo significant volumetric changes which are affected by the graft source and functional loading patterns. Iliac crest bone grafts, which were used for maxillofacial reconstruction, showed a mean resorption rate of 87% in mandibular grafts and complete resorption of the maxillary grafts after 6 years (Sbordone et al.

2012). Different studies have reported variable resorption rates for iliac crest bone grafts, the resorption rate was higher during the first 3-6 months post-grafting (24.16%-31.42%) (Cansiz et al. 2020, Mertens et al. 2013). NABGs from calvarium showed a more favorable results with an average 6-months resorption rate of 8.44%-16.2% (Mertens et al. 2013, Smolka et al. 2006). Functional loading of the grafted bone with osseointegrated dental implants can slightly reduce the overall resorption rate (Cansiz et al. 2020).

2.6.2.5 Allogeneic bone grafts (Allografts)

An allogeneic bone graft is transplanted from a genetically nonidentical human donor after industrially removing/denaturing of its organic components and subsequent sterilization. Extensive processing steps for removing the cellular and antigenic components aim at overcoming the risk of disease transmission, e.g., HIV and Hepatitis B and C viruses. However, the rigorous donor selection and strict exclusion criteria, are critical to decrease the risk for disease transmission by allograft bone (Buck et al. 1989, Oppenheimer et al. 2008).

Allogeneic bone grafts exclusively have the normal inorganic bone matrix. Their extensive processing and preservation not only deactivate the osteoinductive factors in donor bone, but also decrease its strength and mechanical properties. The use of allografts in craniomaxillofacial reconstruction is limited (Neumann and Kevenhoerster 2009, Oppenheimer et al. 2008).

2.6.2.6 Demineralized bone matrix (DBM)

DBM is produced by the acidification/demineralization of bone from human donors, DBM is a composite of the bone inorganic matrix (mostly type I collagen), growth factors (e.g., BMPs), residual mineral calcium phosphate (1-6%), and some cellular debris (Gruskin et al. 2012). DBM has osteoconductive and osteoinductive properties. This commercially available biomaterials/medical devices come in various forms and sizes, the bone augmenting properties of DBM can vary among different commercial lots and preparations (Bae et al. 2006). The production process of DBM renders it void of viable cells. Recently, novel processing techniques were employed to produce cryopreserved DBM which maintains osteoblastic lineage cells. This has shown encouraging results in some clinical applications, further trials and validation for long-term outcomes are needed (Divi and Mikhael 2017, Shahrdar et al. 2020).

Some DBM preparations, with some degree of mechanical stiffness, have shown encouraging results in reconstructing cranial vault defects (Salyer et al. 1992). Most DBM products are manufactured as bone fillers, which limit their applications in large craniomaxillofacial reconstruction. In addition to the batch variability and available-form limitation, the use of DBM in craniomaxillofacial reconstruction showed a great dependence on the surrounding tissue envelope and a significant degree of unpredictable resorption (Gruskin et al. 2012, Ousterhout 1985).

2.6.2.7 Xenogeneic bone grafts

The use of xenogeneic bone grafts has been first reported in 1632 when a Russian soldier received a bone transplantation from a dog by the Dutch surgeon Job Van Meek'ren (Gruskin et al. 2012). Two centuries later, in 1859, Adolf Bardeleben revived the use of xenogeneic bone grafts. Currently, these grafts are produced by a similar process to that of allografts and usually derived from a bovine source. Xenogeneic bone grafts carry concerns of immunogenicity and transmission of infectious diseases. They have been, however, actively used for many years in dental implant surgery and small bony defects (Neumann and Kevenhoerster 2009).

2.6.2.8 Alloplastic biomaterials

The history of biomaterials' evolution extends over more than three millennia of human history. The Edwin Smith papyrus (1500-1600 BC), which attempted to record the knowledge of that time's previous generations, documented the use of sutures for closing wounds among other treatments (Marin et al. 2020, Sullivan 1996). Since the introduction of the field of *"Biomaterials"* in the early 1960s, the definition of a *"biomaterial"* has been updated frequently (Marin et al. 2020). Earlier definitions generally described biomaterials as any material used as an implant in a living system and being inert substances (Cohen 1967, Marin et al. 2020). Obviously, those definitions have prioritized the absence of complications after *in vivo* application without considering the potential favorable bioactive responses. Later definitions in the 1980s have expanded to include any non-viable material, excluding drugs, which interact with biological systems for any period of time to achieve a biomedical function (Patel and Gohil 2012, Williams 1987).

In 1991, during the "Consensus Conference" of Chester, United Kingdom, the most widely accepted definition was introduced: *"Any substance or combination of substances, other than drugs, synthetic or natural in origin, which can be used for any period of time, which augments or replaces partially or totally any tissue, organ or function of the body, in order to maintain or improve the quality of life of the individual"*. Despite being wide enough to cover all the possible applications, this definition is too broad and has been criticized for relying on a relative concept of *the quality of life* (Marin et al. 2020).

Equally confusing are the effective specifications of the biomaterials which should be used for clinically translatable regenerative approaches. The biomaterial selection has been dominated by the requirements for biodegradability and prior United States Food and Drug Administration (FDA) approval which would favor maximum chemical and biological inertness. Such approach should be revisited as it is counter-productive and can endanger the sustainability of innovative regenerative approaches (Williams 2019). In the following subsections, currently available biomaterials will be presented in the context of craniomaxillofacial bone reconstructive applications.

Titanium

Bridging of craniomaxillofacial bone defects using metal alloplastic implants could provide a stable three-dimensional reconstruction with a universal applicability and availability with no donor-site morbidity (Kuttenberger and Hardt 2001). Titanium has been effectively replacing other metals in both osteosynthesis and reconstructive applications since its superior biocompatibility and favorable mechanical properties was demonstrated by Brånemark in 1983 (Branemark 1983, Neumann and Kevenhoerster 2009). Its modulus of elasticity is favorable for less stress-shielding effects and it is highly stable, albeit light weight (Neumann and Kevenhoerster 2009).

The electrochemical properties (corrosion resistance) of titanium alloys considerably modulate their *in vivo* biological behavior and bone-implant interaction. Such interaction is dictated by the composition and properties of the nano-scaled formed surface titanium oxide layer (Dias Corpa Tardelli et al. 2020). Currently, research efforts are devoted for controlling this corrosive phenomenon by optimizing the chemical composition of titanium alloys and their surface treatment. The aim is to achieve adequately thick and firmly adhered surface oxide layer to allow osseointegration with minimal release of implant ions to the surrounding tissues or distant organs which is an increasing concern with the most widely used Ti-6Al-4V (Catalani et al. 2013, Dias Corpa Tardelli et al. 2020, Mirza et al. 2017, Moretti et al. 2012). Recently, beta titanium alloys have been proposed as a promising alternative with a more favorable biocompatibility, however, more studies are still needed for validating its long-term health effects in comparison with Ti-6Al-4V (Dias Corpa Tardelli et al. 2020).

Titanium meshes has provided encouraging results in craniofacial reconstruction of non-load-bearing areas. Those meshes are supplied in different thicknesses to serve in different clinical scenarios. Thick meshes (0.3 and 0.6 mm) can treat the contour irregularities due to a cranial traumatic defect of less than 25 cm^2, whereas thinner meshes (0.1 mm) can be used for repairing frontal sinus anterior table (Kuttenberger and Hardt 2001). A recent systematic review, however, showed that titanium alloplastic cranioplasty reconstruction was associated with local complications (13.09%), infection (6.02%), and alloplastic graft failure (6.02%) (Oliver et al. 2019). Titanium meshes are generally not recommended with poor soft tissue condition and with radiotherapy (Kuttenberger and Hardt 2001, Neumann and Kevenhoerster 2009).

Titanium meshes have been applied as a containment for various particulate bone grafts in maxillary and mandibular alveolar reconstruction. In this context, the titanium mesh intraoral exposure rate was reported to be as high as 52%, thus, tension-free wound closure is critical to decrease such complication (Louis et al. 2008). Mesh exposure is not necessarily associated with a decreased amount of regenerated bone especially when exposure happens in later healing phases (Louis et al. 2008).

For large mandibular defects, titanium reconstruction plates were employed, in primary reconstruction, with or without pedicled musculocutaneous flaps, with adequate results (Söderholm et al. 1988). As the survival rate of reconstructed oral cancer patients is increasing, the complications of titanium reconstruction plates have been progressively reported. In a case series, Lindqvist and coworkers reported a complication rate of 44% (Lindqvist et al. 1992). Combining the reconstruction plate with soft tissue free flaps was advocated to reduce the risk for plate exposure, however, plate fracture occurs in a considerable percentage, especially in dentate patients (Chepeha et al. 2008). The incidence of plate exposure and failure in reconstructing anterior mandibular defects is higher. Plate reconstruction combined with free soft tissue flap has many late complications, it could be, however, a suitable option in patients with poor prognosis for reconstructing lateral mandibular defects (Boyd et al. 1995, Wei et al. 2003).

Modifications in the design of the titanium plating system has been suggested for decreasing complication rates, e.g., THORP and UniLOCK (Stratec Medical, Oberdorf, Switzerland). Despite the relative better performance of UniLOCK system, both were associated with complications including infection, delayed wound healing, and plate exposure (Gellrich et al. 2004). The MatrixMANDIBLE Preformed Reconstruction Plates (MMPRPs) have shown to reduce the operative time and facilitate a transoral approach, however, complications including plate exposure, plate loosening, and orocutaneous fistulas has been reported in 27.1% of the patients (Probst et al. 2012).

Recently, CAD-CAM patient-specific implants (PSI) has gained an increasing attention for developing individual solutions in selected cases. These digitally engineered implants can offer an innovative platform and rapid solution for dental rehabilitation in patients with severe atrophy or jaw resections (Gellrich et al. 2017). Titanium 3D-printed PSI has been applied for reconstructing large mandibular defects with promising outcomes (Darwich et al. 2020). However, long-term results are still unavailable (Darwich et al. 2020, Gellrich et al. 2017). Obviously, titanium is inherently thermosensitive and offers a limited possibility for intraoperative shaping and adjustment. The use of titanium in craniomaxillofacial reconstruction should involve the careful consideration of the surrounding soft tissue condition (Neumann and Kevenhoerster 2009).

Ceramics

Bioactive glass

The first hypothesis of the bioactive glass was proposed in 1967 to overcome the human-body rejection of metallic and synthetic polymeric materials. Inspired by the bone tissue microstructure, Larry Hench and colleagues, Ray Splinter, Ted Greenlee, and Bill Allen, hypothesized that *"if a material is able to form a hydroxyapatite layer in-vivo, it may not be rejected by the body"* (Hench 2006). After implanting the biomaterial into a rat femoral model for six weeks, Dr. Greenlee reported: *"These*

ceramic implants will not come out of the bone. They are bonded in place. I can push on them, I can shove them, I can hit them and they do not move. The controls easily slide out". These findings and further development led to the first bioactive glass in 1971; Bioglass® 45S5 (45% silicon dioxide, 24.5% sodium oxide, 24.5% calcium oxide and 6% phosphorous oxide) (Hench 2006).

Bioactive glass is highly reactive in liquid media through the formation of silicic acid, which develops a high surface area hydrated silica layer holding the glass particles together. This layer gradually precipitates calcium and phosphate ions from the body fluids to form a calcium phosphate surface layer with subsequent crystallization into hydroxyapatite (Hench et al. 1971). This reaction endows bioactivity and surface-osteoinduction to the bioactive glass with an intensive bond at the bone-implant interface. The clinical effectiveness of bioactive glass and its bonding to bone have been extensively explored by the pioneer work of Orjan Andersson, Kai Karlsson and Antti Yli-Urpo at Åbo Academy and University of Turku, Finland (Hench 2006). In the 1980s, Karlsson and Andersson designed and developed novel S53P4 bioactive glasses by modifying the biomaterial within the 45S5 compositional range. This represented a milestone discovery as the S53P4 exhibited additional profound antibacterial properties (Andersson et al. 1990, Hench 2006, YLI-URPO 1990).

Bioactive glass has been mainly applied in alveolar ridge augmentation and sinus lifting for dental implants, and in periodontal bone regeneration with successful results. Being characteristically unfavorable for microbial growth, bioactive glass has been successfully applied for fronto-orbital and calvarial bone defect reconstruction (Aitasalo and Peltola 2007, Gosain 2004, Piitulainen et al. 2019). Bioactive glass has been criticized for the long-term clinical outcomes in reconstructing large bone defects, which were associated with poor vascularization. This was suggested to be due to the slow degradation rate which would not coincide with the rate of new bone formation. However, a growing evidence shows the potential of bioactive glass for reconstructing large bone defects through a two-stage induced membrane technique with promising results encouraging its application even in a single-stage treatment of bone defects (Björkenheim et al. 2019, Björkenheim et al. 2017). The inherently fragile amorphous glass network renders bioactive glass unsuitable for load-bearing applications (Neumann and Kevenhoerster 2009). Nevertheless, bioactive glass continues to inspire researchers for enhancing its mechanical properties and applications in bone reconstruction by combining with polymer biomaterials (Granel et al. 2019, Vallittu 2017).

Calcium phosphates

Calcium phosphates can be synthesized in the laboratory or derived from natural sources, e.g., corals. Closely resembling the inorganic component of bone matrix, calcium phosphates are generally well-tolerated *in vivo*, they are nontoxic and do not cause inflammatory or foreign body reaction (Neumann and Kevenhoerster 2009,

Oppenheimer et al. 2008). A century ago, Albee first reported the application calcium phosphates in enhancing the repair of bony defects (Albee 1920).

In addition to the similarity in composition to the bone mineral, calcium phosphates are bioactive as they can promote cellular function and expression, forming a surface of bone-like apatite or carbonate-hydroxyapatite, which enables the uniquely strong and direct interface with bone. All calcium phosphates are osteoconductive by providing a scaffold for new bone formation (LeGeros 2002, Neumann and Kevenhoerster 2009). The behavior of different calcium phosphates, e.g., hydroxyapatite, tricalcium phosphates, biphasic calcium phosphates, and non-sintered calcium phosphates, is multifactorial. This is affected by the material solubility, cell-mediated degradation, the material components, and manufacturing (LeGeros 2002, Neumann and Kevenhoerster 2009).

Hydroxyapatite has been in use clinically in the last four decades, it has been already available in the early 1990s as both ceramic porous granules and a non-ceramic cement (Byrd et al. 1993, Costantino et al. 1992). Porous hydroxyapatite granules have achieved good aesthetic outcomes for correcting minor skeletal irregularities and contour deficiencies of the skull, zygomaticomaxillary region, lateral mandible, periorbital area, and temporal region. The reliable augmentation was warranted by the material's poor resorption (Byrd et al. 1993). Despite the unpredictable osteogenic effect, hydroxyapatite cement has an advantage of intraoperative modelling, it has been mostly used for reconstructing calvarial bone defects (Gosain et al. 2004).

Hydroxyapatite ceramics use in craniomaxillofacial area has been limited to low-stress regions due to its inherent brittleness and low flexural and torsional strength (Neumann and Kevenhoerster 2009, Oppenheimer et al. 2008). The long-term results for hydroxyapatite cements in large calvarial defects are not promising, complications were reported in up to 50% of the cases with late complications occurring as late as 6 years postoperatively (Zins et al. 2007).

Beta-tricalcium phosphate (β-TCP) ($Ca_3(PO_4)_2$) resorbs 12-22 times faster than hydroxyapatite ceramics depending on the surrounding pH (Jarcho 1981). This higher rate of resorption is challenging for the biomaterial mechanical stability as it often exceeds the rate of new bone formation. Such disproportion is addressed by combining β-TCP with other osteoinductive agents and controlling the material's porosity (Neumann and Kevenhoerster 2009). β-TCP manufacturing critically affects the material's behavior, different β-TCP ceramics show significant differences regarding their phase purity, primary particle size, stability, porosity, solubility, and biodegradation (Peters and Reif 2004). β-TCP is mostly applied in periodontal and dental implant surgery (Guillaume 2017).

Mixtures in different proportions of hydroxyapatite and β-TCP (biphasic calcium phosphates) have been suggested to introduce macropores into the biomaterial and

enhance the bone ingrowth (Gosain et al. 2004). The formation of bioactive carbonate-hydroxyapatite microcrystals is proportional to the biomaterial dissolution, it is more abundant in the biphasic calcium phosphates with lower hydroxyapatite to beta-tricalcium phosphate ratios (LeGeros 2002). As calcium phosphates generally are inferior to bone in mechanical strength, they are not recommended in voluminous reconstructions and in load-bearing applications and should be applied selectively (Fernandez de Grado et al. 2018).

Calcium sulphate

In 1892, Dressman reported the use of calcium sulphate (plaster of Paris) in filling bone defects, which makes it the oldest ceramic bone graft (Dressman 1892, Neumann and Kevenhoerster 2009). Calcium sulphate has been effective clinically in maxillary sinus augmentation for dental implant insertion (De Leonardis and Pecora 2000). Calcium sulphate's application is challenged by its exothermic setting reaction, sensitivity for fluid contamination during handling, low mechanical properties, and rapid resorption within a couple of months. It serves, however, as a good vehicle for drug release, e.g., antibiotics in cases of osteomyelitis (Englert et al. 2007).

Natural polymers

Natural polymers constitute a heterogenous group of materials with a wide range of properties and applications. For regenerative biomedical applications, these biomaterials include selected proteins (e.g., collagen, silk, and fibrin gels) and polysaccharides (e.g., alginate, chitin/chitosan, starch, heparan sulfate, and hyaluronic acid derivatives). These polymers have superior biocompatibility and bioactivity compared to synthetic polymers (Filippi et al. 2020). Bioactive natural polymers can better mimic ECM by featuring cell recognition and adhesion sites. Nevertheless, their properties and behavior depend on extraction and processing procedures which are usually complex. Additionally, their low stability, potential immunogenicity, and inadequate mechanical properties need to be addressed for wider clinical applications (Bhatia 2016, Salehi-Nik et al. 2017). The advances in natural polymer research is necessary for predictable clinical translation of biomedical regenerative approaches (Filippi et al. 2020, Williams 2019).

Synthetic polymers

Synthetic polymers present an attractive field of materials science with an unlimited potential, they are considered the materials of the 21st century (Voit 2017). Synthetic polymers feature a better control on the design and properties of the material, e.g., degradation kinetics, molecular weight, physical properties, and porosity, as well as the final shape of the implant, i.e., PSI. However, polymer-based bone substitutes are generally unpredictable for promoting new bone growth (Carson and Bostrom 2007, Fernandez de Grado et al. 2018).

Acrylate polymers

These non-resorbable polymers include hard tissue replacement (HTR) implants. HTRs consist of sintered poly-methyl-methacrylate, poly-hydroxyethyl-methacrylate, and calcium hydroxide. They are available in granules, blocks, or preformed implants. PSI-HTR has been successfully used for reconstructing large calvarial bone defects and for craniofacial reconstruction (Eppley 2002). Careful preoperative planning, proper consideration of the quality of covering soft tissue, and absence of present/past infection are keys for successful reconstruction. Despite the favorable physical, mechanical properties, volume stability, and porosity, these implants are not likely to be osteoconductive (Neumann and Kevenhoerster 2009).

Porous polyethylene (PPE)

PPE is a linear highly compressed-sintered aliphatic hydrocarbon. MEDPOR® (Stryker, US), for example, has been an efficient and biocompatible PPE-implant option since 1985. Its characteristic omnidirectional porosity favors fibrovascular ingrowth and integration into the recipient site. It has good working properties which allow shaping, trimming, drilling and screw retention. It is available in different stock-forms and as PSIs for reconstructive and aesthetic augmentations of the calvarium, mandible, chin, zygoma, and orbits (Neumann and Kevenhoerster 2009).

The successful integration of PPE implant is critically affected by the condition of the covering soft tissue. Infection, reported in 6% of the cases, is associated with poor soft tissue condition (Cenzi et al. 2005). Subperiosteal placement of the implant and proper fixation can help the successful integration, however, no bone ingrowth is expected into this inert biomaterial (Tark et al. 2012).

Polyetheretherketone (PEEK)

PEEK is a polyaromatic semi-crystalline high-temperature thermoplastic polymer. It has distinctive physicochemical and mechanical properties which make it interesting in industrial and biomedical applications for replacing metal implant components. PEEK is compatible with reinforcing agents, e.g., glass and carbon fibers. It is inherently inert, which explains its biocompatibility and also explains its limited bioactivity (Kurtz and Devine 2007, Williams et al. 1987).

PEEK has been applied in many clinical applications including spinal cages, orthopedic and hip-replacements, craniomaxillofacial bone replacement, intracardiac pumps, and dental implants (Panayotov et al. 2016). For calvarial bone defects, PEEK-PSIs feature stable results avoiding donor-site morbidity and titanium-thermal conduction (Ng and Nawaz 2014). However, being relatively thick, they need a careful design of the skull incision and proper placement to prevent bulging. Additionally, PEEK implants are not porous enough to allow for drainage of seepage as compared to titanium meshes. A recent review reported an overall success rate of 93.7% for PEEK-cranioplasty, complication rate was 15.4%, most common complications

included reoperation in 7.3% and infection in 6.3% of reported cases (Jibo Zhang et al. 2019).

In maxillofacial applications, PSIs made of PEEK proved to be a reliable option with good aesthetic/functional outcomes. The material allows for some intraoperative trimming/modification, infection rate was reported to be 8.3% (Järvinen et al. 2019). In addition to the need for cost reduction, this unique biomaterial remains the focus of future research for balancing the favorable mechanical properties while improving its bioactivity, porosity, thinning, and bony integration (Jibo Zhang et al. 2019).

Biodegradable synthetic polymers

This category of biomaterials is of special interest for regenerative medicine applications. Biodegradable synthetic polymers offer the possibility to control the mechanical properties and degradation kinetics to suit different applications. In the context of bone regeneration, the ultimate goal is to create a synthetic bioactive bone graft which offers good mechanical properties, while remaining resorbable. Such behavior would favor the ingrowth of new bone and vasculature, thus promoting the biomaterial integration/remodeling with the new bone over time (Carson and Bostrom 2007, Hacker et al. 2019).

For bone-related applications, polyesters have been of prominent importance. The potential of these synthetic polymers has been appreciated since the pioneer work of Langer and Vacanti (Vacanti and Langer 1999). Biodegradable polyesters include poly(lactic-acid) (PLA), poly(glycolic-acid) (PGA), poly(ε-caprolactone) (PCL), and their co-polymers, e.g., poly(lactic-*co*-glycolic acid) (PLGA) (Hacker et al. 2019). PLA, PGA, and PLGA have shown adequate biocompatibility *in vitro* and *in vivo*, they are among the few biodegradable polymers approved by the FDA for clinical applications in human. PCL is biocompatible with a slower degradation rate compared to PLA (Hacker et al. 2019, Salgado et al. 2012).

PLA has a good mechanical strength, however, it is brittle and has a relatively low glass transition temperature (~58°C) (Alizadeh-Osgouei et al. 2019, Mohanty et al. 2005). PLA is environmentally friendly and sustainable. The inherent brittleness and poor thermal resistance of PLA have been overcome by the development of self-reinforced PLA (SR-PLA) or “all-PLA” composites (Mai et al. 2015). This technology implies the stacking of films/fibers of similar polymers with different melting temperatures. Achieving the melting of one phase yields the matrix, while retaining the other phase for reinforcement (Alcock and Peijs 2011). Törmälä and coworkers pioneered the manufacturing and production of SR-PLA for surgical implants after studying their behavior *in vivo* (Kellomäki et al. 2000, Törmälä et al. 2002). SR-PLA plates and screws were efficiently used for internal fixation first in preclinical studies (Suuronen 1991, Suuronen et al. 1998) and later in orthognathic surgery and maxillofacial fractures (Haers et al. 1998, Suuronen 1993, Suuronen et al. 2000, Ylikontiola et al. 2004).

PLA slowly degrades through hydrolysis, it is relatively hydrophobic and lacks reactive side chain groups and is thus chemically inert (Casalini et al. 2019). Because lactic acid is a chiral molecule, PLA has two stereoisomeric forms of its lactide dimer units: -L-lactide (PLLA) and D-lactide (PDLA). PLLA is the semi-crystalline form which has better initial mechanical strength and is more resistant to hydrolysis. *In vivo*, the enzymatic and hydrolytic biodegradation of PLLA produce the naturally occurring L-lactic acid, which enters the physiological metabolic pathways and is excreted from the lungs as water and carbon dioxide (Zeeshan Sheikh et al. 2015).

PGA has a high crystallinity and tensile modulus and a higher degradation rate due to its relative hydrophilicity. PGA degrades into hydroxyacetic acid which undergoes hepatic metabolism or renal excretion. The mechanical strength of PGA deteriorates rapidly within few weeks post-implantation *in vivo,* which challenges its application in large or load-bearing bone defects (Zeeshan Sheikh et al. 2015).

Copolymerization of PLA and PGA has been advocated to tune the properties of both materials, this has introduced different forms of PLGA. Combining different ratios of the monomers allows the control of hydration and hydrolysis. Apart from the 1:1 ratio of PGA:PLA, which shows the fastest degradation, the more glycolides, the quicker the rate of degradation. Although PLGA has been considered promising in bone regeneration applications, its use is limited to small and contained defects due to the relatively poor mechanical properties (Campana et al. 2014, Zeeshan Sheikh et al. 2015).

PCL is a semi-crystalline polyester which is highly processable due to its solubility in a wide range of organic solvents. It is a hydrophobic polymer which hinders the water intrusion and subsequent degradation by hydrolysis (Campana et al. 2014, Salgado et al. 2012). These polymers are regarded as promising and biocompatible, however, their slow degradation rate and inferior mechanical properties require improvement (Zeeshan Sheikh et al. 2015).

Generally, polyesters undergo biodegradation via bulk erosion (Figure 2.5), in which the diffusion rate commonly exceeds the hydrolysis rate. This leads to an abrupt mass loss after a certain time interval. Additionally, the accumulation of acidic oligomers in the core of the material, leads to a faster degradation in the bulk compared to the surface, such phenomenon is called autocatalysis (Figure 2.5) (Casalini et al. 2019). Obviously, the relatively sudden mass loss translates into a rapid deterioration of the mechanical properties, which should be critically considered in bone regeneration applications.

The degradation behavior of polyesters raises additional concerns related to the accumulation of the acidic degradation products within the bulk of the material. This detrimentally affects the local environment and can cause late noninfectious inflammatory response when degradation products are burst-released upon structure breakdown (Hacker et al. 2019). This is especially concerning in

craniomaxillofacial reconstructions which require large-sized implants covered by relatively thin tissues. In summary, limitations to this category of synthetic polymers are related to their low bioactivity, insufficient mechanical properties for load-bearing applications, and the critical degradation pattern and the acidic degradation products (Hacker et al. 2019, Webb et al. 2004).

Polycarbonates have emerged as interesting biomaterials with favorably controllable mechanical properties and good processability. Poly(trimethylene carbonate) (PTMC) is the most prototypical polycarbonate (Hacker et al. 2019). It is a highly biocompatible and biodegradable aliphatic polycarbonate. PTMC stands out with a unique degradation behavior, unlike aliphatic polyesters, it is resistant to non-enzymatic hydrolysis, undergoes enzymatic degradation with surface erosion (Figure 2.5), and its degradation products are non-acidic (Fukushima 2016, Zhang et al. 2006).

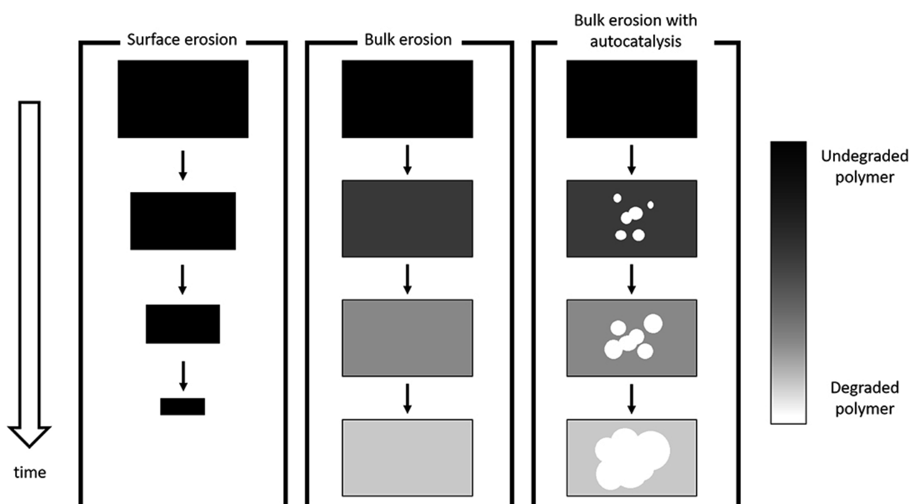

Figure 2.5: Schematic representation for the degradation mechanisms. Surface erosion is seen with poly(trimethylene carbonate) (PTMC), whereas bulk erosion with potential autocatalysis occurs with polyesters, e.g., PLA and PGA. Reproduced from (Casalini et al. 2019) article under Creative Commons Attribution License (CC BY)

PTMC is flexible and soft, however, its mechanical properties can be tuned by changing its molecular weight, crosslinking, and copolymerization. These mechanisms can also control the rate of biodegradation of PTMC, e.g., high molecular weight PTMC degrades faster *in vivo* compared to low molecular weight PTMC. Additionally, the pendant side groups within the structure of these polymers allow not only a better control of its mechanical/biodegradation behavior, but also on their bioactivity and cellular responses. Incorporation of bioactive ceramics within PTMC can also enhance its toughness as well as osteoconductive effect (Fukushima 2016, Hacker et al. 2019, Teotia et al. 2020).

Polymer-ceramic composite biomaterials

Composites of polymers and bioactive inorganic ceramics have attracted much interest with the aim of optimizing the material's mechanical properties and improving bioactivity. These composites are also of interest for customizable reconstructive approaches using CAD-CAM and 3D printing technology (Li et al. 2014).

Composites of PLA and nano-HA (50:50 wt %) have proven to be osteoinductive by inducing heterotopic bone formation in paraspinal muscles of dogs. Additionally, degradation of the composites was reduced as compared to the non-bioactive PLA control (Danoux et al. 2014). The weak bonding between HA and PLA, however, should be addressed for a better performance (Alizadeh-Osgouei et al. 2019, Hong et al. 2005). PLA-bioactive glass (45S5 BG®) composites have shown that a larger bioactive glass content led to an increase of the bioactive HA crystallization at the surface, however, it also led to a more rapid degradation. The best balance between bioactivity and stability of the material was estimated to be achieved with 30% 45S5 BG® (Turnbull et al. 2018).

PCL/HA composites have been shown to be suitable for fabricating scaffolds of specific geometry using selective laser sintering (SLS) technology (Wiria et al. 2007). This technology allows not only the production of customized complex anatomical parts, but also the improvement of the mechanical properties over pure PCL (Eshraghi and Das 2012).

Composite porous scaffolds were produced from PLGA and nano-HA via SLS were reported to allow a better control over the pore architecture and exposure of the bioactive HA at the surface. The mechanical properties were enhanced by adding up to 20 wt % of the nano-HA content, however, further increase of the ceramic content sharply deteriorated the mechanical properties (Shuai et al. 2013). A similar observation is true for non-degradable polymers, for example, loading PEEK with 40% HA has decreased the ultimate tensile strength of the material by 45%, the weak link lies at the polymer-HA interface (Kurtz and Devine 2007).

Interestingly, Guillaume and coworkers has applied stereolithography (SLA) for producing PSI made of PTMC loaded with 40 wt % of HA. The PTMC-HA composites have shown osteoinduction ectopically and successful reconstruction for orbital floor defects in a sheep model (Guillaume et al. 2020). Dienel and coworkers have optimized SLA additive manufacturing for PSIs made of PTMC with high amounts of β-TCP (60 wt %). Manufacturing of a large PSI was successful at a high resolution using composites of PTMC and 51 wt % β-TCP, with β-TCP readily available at the implant surface (Dienel et al. 2020). These SLA-fabricated PTMC-ceramic composites has demonstrated favorable osteoconductivity in treating calvarial and tibial CSD in a rabbit model (Teotia et al. 2020). Given the favorable degradation behavior of PTMC over polyesters, PTMC composites are gaining appreciation as the next

generation synthetic bone substitutes. Such potential warrants further analysis and testing in suitable preclinical models.

In summary, despite the diversity of alloplastic biomaterials, the ideal bone substitute, which is biocompatible, inexpensive, biodegradable, fully osteoconductive, can be used for PSI manufacturing, and has favorable mechanical properties, remains elusive. When selecting the appropriate biomaterial, the mechanical stresses to the biomaterial must be considered. It is, so far, a *trade-off approach* since favorable mechanical properties are compromised with an increase of osteoconductivity and biodegradability (Neumann and Kevenhoerster 2009, Oppenheimer et al. 2008). However, the search for more predictable options is active and ongoing.

2.7 Tissue engineering and regenerative medicine

2.7.1 Terms and concepts

Generally, tissue engineering/bioengineered tissues and regenerative medicine are frequently used interchangeably for indicating the replacement, repair, and/or regeneration of tissues and organs (Nerem 2006). These concepts represent the hallmarks for the current era, in which we are trying to approach the problems of failing tissues and organs by imitating nature and harnessing the intrinsic power of biology (Nerem 2006). Obviously, the potential is too broad to envisage, hence, researchers are exploring the field with mixed approaches. Therefore, it is important to present key terms and related concepts.

Our current conceptualization for tissue engineering has started in the late 1980s. However, much earlier intuitive statements date back to 1938; in a book, by Alexis Carrel and Charles Lindbergh, entitled: “The culture of organs”. The authors presented some of the basic concepts/statements which holds true in our current approaches, e.g., *“Anatomical specimens are nothing but useful artefacts”, “…structure and function have no separate existence”, and “Cells and medium are one”* (Carrel and Lindbergh 1938, Nerem 2006). Even earlier, the relation between the structure of cancellous bone and mechanical stresses was already emphasized during 1860s by the interaction of Georg Hermann von Meyer, a distinguished anatomist, with Karl Culmann, a famous structural engineer and mathematician. Inspired by the bone structure and the loading pattern of the human femur, Culmann with his student constructed the principal stress trajectories in a similarly curved crane-like bar which later became the famous “Culmann’s crane”. These observations paved way for the development of the trajectorial hypothesis of bone adaptation which is the famous “Wolff’s law” by Julius Wolff. However, the concept that bone cells can adapt to the local mechanical stresses and to new environments should be credited to Wilhelm Roux in his “Functional adaptation concept” (Fung 1990, Kivell 2016, Skedros and Brand 2011).

Tissue engineering was initially developed as a branch of biomaterial science, specifically focusing on polymer chemistry and the interaction between the cells and the newly developed biodegradable materials (Saxena 2017, Vacanti et al. 1988). In 1987, the term "tissue engineering" was devised in a committee meeting of the National Science Foundation. A subsequent tissue engineering first time workshop was held by the Lake Tahoe, California, in early 1988, which provided the first definition. Skalak and Fox have reported the proposed definition of tissue engineering as "…*the application of principles and methods of engineering and life sciences toward the fundamental understanding of structure-function relationships in normal and pathological mammalian tissues and the development of biological substitutes to restore, maintain, or improve tissue functions*" (Skalak and Fox 1988).

As the concept and practice of the field has further been developed, the central role of cell biology and culture methods in the tissue engineering strategies has been growingly emphasized (Langer and Vacanti 1993, Skalak 1993). It is currently accepted that tissue engineering involves the use of relevant scaffold material, appropriate growth factors/bioactive molecules, and/or the appropriate cells for developing new therapies (Tang et al. 2016).

The term "regenerative medicine" has been popularized during the 1990s, and it is considered to non-exclusively refer to stem cell technology (Nerem 2006). Regenerative medicine includes tissue engineering as one of its scientific platforms, it aims to develop strategies which foster repair and regeneration and hopefully bypass the need for tissue replacement (Nerem 2006, Williams 2019).

It is crucial to highlight the conception of *engineering* in tissue engineering as the profound link between engineering systems and regenerative medicine. As argued by David F. Williams, it is true that *engineering* can reflect the use of scientific physical data to solve practical problems and yield an end-product, however, the underlying science for tissue engineering's end-product is more related to developmental, cellular, and molecular biology, as well as to pharmacology (Williams 2006). Therefore, the *engineering* here can be best described by its Latin origin: *'ingenium'*, essentially, referring to the creativeness. This creative process in tissue engineering is mediated by living cells/tissues which are stimulated into an unnatural regenerative mode by either biomolecules (molecular signals) and/or the supporting structures (mechanical signals) (Williams 2006).

Regeneration should be appreciated as the essence of tissue engineering, which involves the persuasion of the body to heal itself through the delivery of cells, biomolecules, and/or supporting structures, to the appropriate site (Williams 1999, Williams 2006). Hence, Williams suggested a more precise conceptual definition for tissue engineering which is *"…the creation of new tissue for the therapeutic reconstruction of the human body, by the deliberate and controlled stimulation of selected target cells, through a systematic combination of molecular and mechanical signals"* (Williams 2006).

2.7.2 Bone tissue engineering (BTE) elements and clinical challenges

Tissue engineering concepts have been applied experimentally for generating different tissues including bone tissue (Kneser et al. 2006, Langer and Vacanti 2016, Vacanti and Vacanti 1994). It is important to dissect the potential components of the tissue engineered construct; however, all the components interact together. Essentially, our understanding of such an interaction is a prerequisite for a sustainable BTE (Williams 2019).

2.7.2.1 The matrix equivalents

In normal bone tissue, the ECM is not only responsible for the bone mechanical properties, but also serves as the biological platform for the cells, providing them with the tissue-specific environment and architecture. The cellular adhesion to ECM is a determinant for cell shape and mechanical signaling in the cytoskeleton, which controls cell-cycle progression and activity (Huang and Ingber 1999). Additionally, bone ECM acts as a reservoir for minerals, water, nutrients, cytokines, and growth factors (Kneser et al. 2006).

In the context of BTE, the research for an equivalent, or probably a semi-equivalent, of bone ECM is in the core of any BTE approach. Essentially, this tacitly involves the use of biomaterials as vehicles for the controlled delivery of the desired molecular and mechanical signals to the selected target cells/tissues (Williams 2019). These biomaterials have been routinely referred to as scaffolds, which, probably, does not optimally describe their aimed role. Williams has considered that the concept of a scaffold biomaterial which guides tissue regeneration is too simple to result in sustainable tissue engineering approaches. A scaffold would indicate the provision of a mechanical facilitation for the *building* of a construct, followed by its disassembly and removal. On the contrary, biomaterials in BTE are rather expected to play an active role in the *building* process, which can be better described as a template. Hence, Williams suggested that the BTE template is *"a biomaterials-based structure of defined size, chemistry and architecture that controls the delivery of molecular and mechanical signals to target cells in tissue engineering processes"* (Williams 2019).

Among the various interconnected functions of the ECM, a BTE template is aimed to provide an adequate mechanical support, cellular attachment, and interaction in cellular communication/response (Kneser et al. 2002, Kneser et al. 2006). Various biomaterials, including those discussed in earlier sections, have been employed as BTE templates (Shin et al. 2003, Yang et al. 2002). Fibrin, among other gel-like biomaterials, has been applied for cell immobilization in BTE constructs (Kneser et al. 2005). The rationale behind the biomaterial selection should be carefully evaluated, which necessitates a look into the evolution of the biomaterials. In the context of BTE, the development of biomaterials has evolved through three generations, first biomaterials generation is bioinert with suitable physical properties which includes metals (e.g., titanium alloys), some synthetic polymers (e.g., PEEK), and some ceramics (e.g., zirconia). The second-generation includes bioactive materials and

some biodegradable materials (e.g., naturally derived and synthetic biodegradable polymers, calcium phosphates, and bioactive glasses). The third-generation biomaterials can instruct and modulate cellular behavior into a favorable bioresponse while maintaining an optimized performance of earlier biomaterials (e.g., bioactivated composites, growth factor loaded, and nanotechnology-based templates) (Qu et al. 2019). In summary, biomaterials research advances towards improving functionality, optimizing mechanical properties and degradation kinetics, and providing versatile solutions for better customization.

2.7.2.2 Cells

The osteoblast lineage cells have been an integral part of almost any BTE approach. Physiologically, the bone-forming osteoblasts are short-lived and MSCs continuously provide a replenishing source from nearby bone marrow, periosteum, and endosteum. MSCs have been in the focus of many research studies for enhancing bone regeneration (Marolt Presen et al. 2019).

Originally, Friedenstein and coworkers, in the 1960s, described a fibroblast-like subpopulation of cells from the bone marrow while reporting the interaction between different bone marrow elements to form osseous tissue after subcutaneous transplantation (Friedenstein et al. 1966). In 1991, Caplan coined the name "mesenchymal stem cells" to describe mesenchymal tissue cells which are, *theoretically,* capable of differentiating into a variety of end-stage phenotypes. Marrow mesenchymal cells were purified by their differential adhesion to culture dishes, retaining their capacity to differentiate into osteoblasts or chondrocytes (Caplan 1991). However, the cultured plastic-adherent mesenchymal cells could be heterogenous regarding their potencies and contribution to overlapping lineages, or even possibly represent a population of different distinct stem cells (Chan et al. 2018).

According to the position statement of the International Society for Cellular Therapy (ISCT) in 2006, MSCs generally should show the minimal criteria of the capacity to adhere and grow on tissue culture plastic surface, cell-surface expression of CD90, CD105 and CD73, and lack of cell surface CD45, CD34, CD14, CD79 and HLA-DR, and the ability to undergo differentiation into osteogenic, chondrogenic, and adipogenic lineages under standard *in vitro* conditions (Dominici et al. 2006).

Interestingly, MSCs, which are isolated from different tissues via *in vitro* culturing, have been relatively poorly characterized regarding their *in vivo* phenotype, natural history, native identity, and natural function (Murray and Péault 2015). Recently, Chan and coworkers suggested the identity of genuine, self-replicating multipotent *putative* human skeletal stem cell, which generates progenitors of bone, cartilage, and stroma, but not fat (Chan et al. 2018). A growing research into the *in vivo* origin and behavior of MSCs is warranted. This is expected to advance the understanding

which may allow us to intentionally recruit MSCs into an acellular implant *in vivo*, and harness the regenerative potential of autologous cells (Murray and Péault 2015).

MSCs multiplicity

MSCs have been initially described to reside in bone marrow, however, MSC-like cells have also been isolated from other tissues which include adipose tissue, dental pulp, dental follicle, and umbilical cord. Tissues which are considered as medical waste, e.g., lipoaspirates, are usually attractive sources for MSCs (Marolt Presen et al. 2019, Wilson et al. 2019).

MSCs from different source tissues can show different behavior. Bone marrow and adipose tissue are among the most employed sources. Bone marrow MSCs (BMSCs) are relatively rare (<0.01%) among the isolated mononuclear cell fraction. Extended *in vitro* expansion for obtaining MSCs numbers for therapeutic use should be weighed against the corresponding decline in their proliferation and differentiation potential over passaging. Depending on the harvesting procedure and site, adipose tissue-derived MSCs (AT-MSCs) can be as high as 1-5% of the isolated nucleated cells. *In vivo* osteogenesis of AT-MSCs is less efficient compared to BMSCs, however, AT-MSCs contain a vasculogenic subpopulation which could be advantageous in the context of bone healing by promoting neovascularization (Brennan et al. 2017, Marolt Presen et al. 2019).

In addition to the source tissue dependent MSC heterogeneity, MSCs from the same tissue show considerable variation between different donors. Additionally, the necessary *in vitro* expansion of MSCs adds another level of heterogeneity, which necessitates a deeper understanding of the mechanisms behind the effects of different environmental cues (Phinney 2012). Such multilevel heterogeneity of MSCs complicates their appropriate characterization and predictable translation into the clinic (Wilson et al. 2019).

The efforts for providing consistency to MSCs as a clinical product has steered the attention to human induced pluripotent stem cells-derived MSCs (hiPSCs-MSCs). The hiPSCs are derived by nuclear programming of adult somatic cells using cocktails of transcriptional factors and/or small molecules working on the pluripotency regulation network, hiPSCs can offer a virtually limitless supply of MSCs (Ma et al. 2013, Marolt Presen et al. 2019).

The hiPSCs-MSCs potentially could preclude the need for continuous tissue donations and alleviate inter-donor variability among other sources of MSCs heterogeneity. Such approach would allow for an off-the-shelf product which is more homogenous, thus clinically translatable as reported recently in the first phase I clinical trial using iPSC-derived MSCs for treating steroid-resistant acute graft versus host disease using Cynata Therapeutics Cymerus™CYP-001 product (Bloor et al. 2018). However, hiPSC-MSCs largely resemble adult MSCs in their surface antigen expression, a point that should be considered in the allogeneic setting as MSCs are not actually immune

privileged (Ankrum et al. 2014, De Peppo et al. 2013). Comparative studies of autologous and allogeneic hiPSC-MSCs, as well as with adult MSCs are still required (Wilson et al. 2019).

MSCs therapy for craniomaxillofacial defects

MSCs have been applied clinically with the basis of providing substitution for the endogenous compromised cells at an injured recipient site. This is based on the hypothesized MSCs stemness/progenitor function *in vivo*. Autologous MSCs have been *in vitro* expanded and combined with biomaterials for treating various craniomaxillofacial bone defects with variable results. While clinical results were not optimal for cranial defects, MSCs treatment groups have shown a trend for higher bone formation in alveolar cleft defects, treatment of maxillary cystic bone defects, and severely atrophied mandibular bone (Gjerde et al. 2018, Khojasteh et al. 2017, Redondo et al. 2018, Sándor et al. 2014, Thesleff et al. 2017).

Several published reports and clinical studies verified the safety of MSCs-based therapeutic approaches and their relative potential for enhancing bone healing depending on the clinical application (Chanchareonsook et al. 2014, Marolt Presen et al. 2019). Nevertheless, safety concerns related to the *in vivo* behavior of the transplanted MSCs and their untoward differentiation are still valid (Harrell et al. 2019). In a cardiac infarction mouse model, transplanted BMSCs were reported to underlie an extended ossification in the infarcted myocardium (Breitbach et al. 2007). Severe bilateral visual loss has developed in patients who received AT-MSCs intravitreal injection for treating age-related macular degeneration (Kuriyan et al. 2017).

The heterogeneity in the study designs and clinical applications, however, not only compromise the level of evidence, but also hamper drawing solid mechanistic conclusions (Chanchareonsook et al. 2014, Marolt Presen et al. 2019). Although some clinicians may argue that MSCs have shown favorable results and that is all that matters, such an approach is risky and could contribute to the related hype which has led to the current perception of tissue engineering being overpromised and under delivered (Nerem 2006).

The limited understanding of the mechanisms behind MSCs therapeutic potential and their fate *in vivo* hampers the effort for predictable therapies (Manassero et al. 2016). For therapeutic preparations, it is difficult to define the optimal MSCs numbers, differentiation stage, the mechanical properties for the engineered constructs, and their bioactivity (Marolt Presen et al. 2019, Oryan et al. 2017). Without the necessary mastering and validation for those factors, researchers approaches could be disastrous as reported recently in the field of tracheobronchial tissue engineering (Williams 2019). No MSCs-based product has become advocated as a predictable treatment for bone defects so far. Future research is required to

advance our understanding of MSCs *in vivo* behavior and therapeutic potential mechanisms (Marolt Presen et al. 2019, Wilson et al. 2019).

MSCs as medicinal cell factories

Currently, the exact mechanism of the contribution of transplanted MSCs to the new tissue formation is not yet elucidated. Limited numbers of transplanted MSCs have shown to survive *in vivo* and engraft at the defect site. Such observations combined with the limited knowledge of MSCs *in vivo* nature and behavior have been fueling the current appreciation of MSCs exerting their therapeutic effects via a so-called "hit and run" mechanism (Ankrum et al. 2014, Geuze et al. 2010, Oryan et al. 2017, von Bahr et al. 2012). This mechanism involves the secretome of the transplanted MSCs mediating paracrine effects on the endogenous cell populations and local environment (Figure 2.6) (Caplan and Correa 2011, Marolt Presen et al. 2019).

Some researches see the lack of sustained engraftment of MSCs as a merit which makes MSCs even safer by limiting their long-term risk (von Bahr et al. 2012). It should be noted, however, that the longer survival of MSCs *in vivo* is associated with better therapeutic effect, which could be largely through their paracrine effects (Geuze et al. 2010, Marolt Presen et al. 2019). The research into the secretome of MSCs will not only augment our understanding for the mechanisms behind MSCs therapeutic potential and interactions, but also open new horizons for better cell-free therapeutic approaches. The secretome-based approaches can be advantageous over other cell- and tissue-based approaches in improving the patient safety profile in allogeneic settings. Additionally, the absence of replicating allogeneic cells, potentially easier quality control, the simple and cost-effective storage, and the potential availability off-the-shelf are promising merits for clinically translatable therapeutics (Marolt Presen et al. 2019).

Extracellular vesicles (EVs)

Our current consciousness for the intercellular communication of MSCs has appreciated the important role of MSCs extracellular vesicles (EVs) (Figure 2.6). EVs comprise a heterogenous group of small (30-2000 nm), lipid-bilayer enveloped, cell-derived particles which include exosomes, microvesicles, and other heterogeneous vesicles (Lamichhane et al. 2014). Interestingly, for long time, EVs have been considered as cellular debris, but recently EVs have been shown to play an active role in fundamental cellular functions and processes (Andaloussi et al. 2013, Lai et al. 2010, Marolt Presen et al. 2019, Sahoo et al. 2011).

Cells secrete EVs to alter the activity of neighboring or distant target cells by the horizontal transfer of proteins, lipids, mRNAs, miRNAs, and other non-coding RNAs. EV lipid bilayer protects these elements from degradation in the extracellular environment, thus making EVs an interesting therapeutic platform for immune response modulation and tissue regeneration (Marolt Presen et al. 2019, Van Niel et al. 2018).

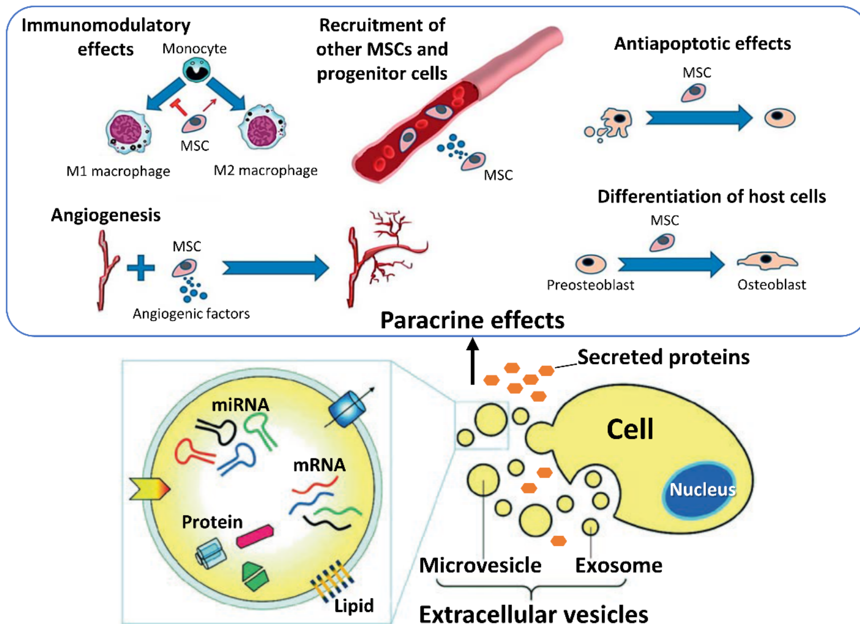

Figure 2.6: Schematic representation for the MSCs secretome (highlighting EVs) and its potential mechanisms of action on bone reconstruction and repair. Adapted with permission from (Kaur et al. 2019, Oryan et al. 2017).

EVs from BMSCs, umbilical cord MSCs, endothelial progenitor cells, and iPSC-MSCs have been shown to enhance bone healing in rodent models (Furuta et al. 2016, Jia et al. 2019, Qi et al. 2016, Yuntong Zhang et al. 2019). The favorable outcomes were associated with promoted angiogenesis (Jia et al. 2019, Qi et al. 2016, Yuntong Zhang et al. 2019). AT-MSCs-derived EVs have been immobilized onto PLGA and were shown to enhance bone regeneration in calvarial defects in mice (Wenyue Li et al. 2018).

Although the mechanisms of the osteogenic effects of EVs are not clearly defined, MSCs-EVs were reported to promote bone repair via pleiotropic effects (Marolt Presen et al. 2019). Those effects include the recruitment of endogenous MSCs, proangiogenic HIF-1α-dependent activity, osteoprotective promotion of angiogenesis, and the direct activation of osteogenic differentiation via miRNA-196a (Furuta et al. 2016, Liu et al. 2017, Qin et al. 2016, Yuntong Zhang et al. 2019). However, careful interpretation of those mechanisms in the context of employed cells and experimental applications is essential (Harrell et al. 2019, Marolt Presen et al. 2019).

In addition to the aforementioned advantages of the cell-free based therapies, EV-based therapies feature a promising translational platform as MSCs-EVs can have the same therapeutic effects of MSCs, are easier to handle/store, stable, and can be sterile filtered (Kaur et al. 2019). However, researchers should strive to address many

relevant challenges. Currently, there is no universally optimal EVs isolation method. Isolation of EVs can be performed by ultracentrifugation, density gradient, ultrafiltration, immunoaffinity, precipitation, and microfluidics. The isolation method depends on sample volume, purity, required integrity and yield, processing time and available equipment (Nawaz et al. 2014). It is possible that different isolation methods yield different EV-subtypes, this should be carefully characterized (Kaur et al. 2019). Essential attention should be focused on the standardization of the cell culture conditions which significantly affect the yield and the cargo of MSCs-EVs (Kaur et al. 2019). The stability of the EVs upon different storage conditions should be quantified and confirmed by quality testing. MSCs-EVs advancement towards clinical applications should be supported by predefined quantifiable metrics which serve to identify EV's cellular origin, presence of lipid-membrane vesicles, and their degree of physical and biochemical integrity. For that purpose, a well-characterized MSCs-EV's biological reference is needed. Much research should be devoted for the proper harmonization of protocols and characterization of MSCs-EVs from different preparations (Marolt Presen et al. 2019, Witwer et al. 2019).

2.7.2.3 The growth factors/bioactive molecules

Growth factors are essential orchestrators of the normal bone regeneration process through mediating intercellular communication and responses. They refer to the class of complex polypeptides which bind to specific transmembrane receptors on the surface of their target cells initiating intracellular signaling pathways. The discovery of the osteoinductive BMPs in 1965 by Marshall Urist is a landmark in the research and development for bone growth factor therapy (Kneser et al. 2006, Le et al. 2017, Nyberg et al. 2016).

In the context of BTE, growth factors are applied as either a crude and hardly standardized mixtures of proteins, e.g., platelet rich plasma and DBM, or as isolated purified factors (Kneser et al. 2006). Growth factors for BTE applications include BMPs, VEGF, fibroblast growth factor (FGF-2), platelet-derived growth factor (PDGF), insulin-like growth factors (IGF), and transforming growth factor beta (TGF-β). BMPs have received great attention in clinical applications due to their robust osteoinductive properties even in ectopic sites (Eğri and Eczacıoğlu 2017, Schliephake and Böhrnsen 2019). VEGF has been of special interest for inducing therapeutic vascularization in the infarcted heart, thus inspiring its application in the vascularization of BTE constructs (Mastrullo et al. 2020, Simón-Yarza et al. 2012).

Recombinant human BMPs (rhBMPs) and VEGF

Recombinant human BMPs (rhBMPs), namely rhBMP-2 and rhBMP-7 have been applied in clinical settings (El Bialy et al. 2017). Currently, only rhBMP-2 is commercially available after rhBMP-7 was discontinued in 2014. The rhBMP-2 is supplied as a lyophilized product at a concentration of 1.5 mg/ml after reconstitution, with an absorbable collagen sponge (ACS) serving as a carrier for the protein. Initially, in 2002, rhBMP-2 was FDA-approved for spine fusion procedures,

later in 2007 it received approval for oral and maxillofacial applications (El Bialy et al. 2017, McKay et al. 2007). The rhBMPs showed promising results for reconstructing alveolar clefts and mandibular reconstructions in selected patients, nevertheless, the clinical evidence for the efficacy of growth factors in maxillofacial reconstruction is limited (Chanchareonsook et al. 2014, Chenard et al. 2012, Schliephake 2015).

With the increasing use of rhBMP-2 for different orthopedic applications, a series of safety concerns and possible side effects have evolved especially in spine-related applications. Such reports highlighted the relationship between complication rates and increased rhBMP-2 concentrations which is released from the ACS via burst release immediately after application (Hustedt and Blizzard 2014). Additionally, in a multicenter, randomized controlled trial which involved treating patients of degenerative lumbar spine using a high dose of rhBMP-2, it was reported to be associated with an increased risk of new cancer (Carragee et al. 2013). However, experts still see several limitations in the available literature including unreported data and inhered bias which preclude drawing sound scientific conclusions regarding the BMPs' potential carcinogenic effects (El Bialy et al. 2017, Pountos et al. 2014). Current research should focus on acquiring the optimal dose regimen via sustained and controlled release of BMP (El Bialy et al. 2017).

BTE applications face great challenges due to the lack of mature and functional vasculature in large BTE constructs. VEGF, a key regulator for angiogenesis, has been in the focus of BTE vascularization studies which included *in vitro* and *in vivo* experiments (Mastrullo et al. 2020). The clinical translation is challenged by the inherent instability and short half-life of this factor, additionally loading tissues with supraphysiologic concentrations can potentially increase off-target effects (Simón-Yarza et al. 2012). Much research should be devoted for controlling the release kinetics of these critical growth factors (Mastrullo et al. 2020, Simón-Yarza et al. 2012).

Small molecules

The use of small molecules has gained an increasing attention as an appealing alternative for growth factors. Small molecules comprise a wide range of nonpeptide natural or synthetic molecules with low molecular weight and low immunogenicity. These molecules feature some interesting advantages from a clinical translational aspect. Unlike growth factors, small molecule's activity is not dependent on a complex 3D structure, they are very stable, less expensive, and non-immunogenic (Schliephake and Böhrnsen 2019). Small molecules present a new platform for better spatio-temporal control of bioactivation in BTE.

Several *in vitro* and *in vivo* preclinical studies have focused on the osteoinductive potential of selected small molecules, e.g., oxysterols, lovastatin, and simvastatin (Balmayor 2015). Recently, the purinergic receptor pathway has gained attention in bone remodeling process via the effects mediated by adenosine. Dipyridamole,

which is a clinical nucleoside transport inhibitor, can block the cellular uptake of adenosine, thus increasing its extracellular levels to stimulate adenosine A2A receptor. Indirect A2A receptor agonists has shown to enhance bone regeneration as much as BMP-2 (Mediero et al. 2016). Dipyridamole has shown promising results *in vivo* for craniomaxillofacial bone regeneration (Lopez et al. 2019, Maxime M. Wang et al. 2019).

The hypoxia-inducible transcription factor (HIF-1)/VEGF pathway has received a great attention for understanding the mechanistic basis of the cellular response to hypoxia. The 2019 Nobel Prize in Physiology or Medicine was awarded to William G. Kaelin Jr, Sir Peter J. Ratcliffe and Gregg L. Semenza for their discoveries of how cells sense and adapt to oxygen availability. Such process involves oxygen-dependent prolyl hydroxylation of HIF-1α under the control of prolyl hydroxylases (PHD 1, 2, and 3) and an asparaginyl hydroxylase known as Factor Inhibiting HIF-1α (FIH), thus leading to subsequent HIF-1α proteosomal degradation (Figure 2.7) (Qing Zhang et al. 2019).

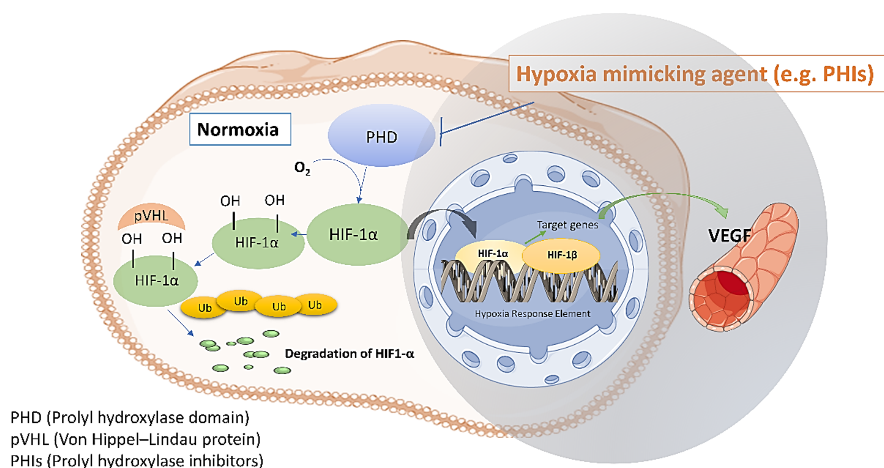

Figure 2.7: Schematic representation for the HIF-1α/VEGF pathway. Adapted with permission from (Maes et al. 2012) and produced using (Servier-Medical-Art) images.

When HIF-1α is stabilized, due to hypoxia or prolyl-hydroxylase inhibitors (PHIs) (Figure 2.7), it translocates into the nucleus to dimerize with HIF-1β to bind hypoxic response element (HRE)-driven promoters on several genes, including *VEGF*, *glucose transporter-1*, *and erythropoietin* (Pagé et al. 2007). The chemical stabilization of HIF-1α by PHIs small molecules can potentially enhance the proangiogenic effects of MSCs for BTE applications. However, different aspects of MSCs response to PHIs are yet to be elucidated.

2.7.3 The *in vivo* bioreactor principle and flap prefabrication technique

Despite the actively increasing numbers of studies on the development of tissue engineered constructs, the delivered clinical solutions are still limited. The lack of mature and functional vascularity within sizable engineered constructs is a key challenge (Mastrullo et al. 2020, Novosel et al. 2011). Our current understanding for the mechanisms and players regulating the angiogenic process *in vivo* has reflected the complex network of interacting factors regulating this phenomenon (Figure 2.8). In BTE applications, such complexity has been only rudimentarily reproduced *in vitro* despite the current advanced technologies (Kasper et al. 2017, Mastrullo et al. 2020).

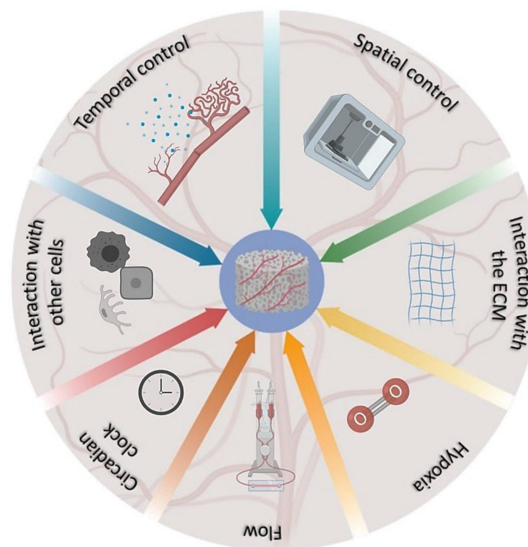

Figure 2.8: Schematic representation for the factors involved in angiogenesis in vivo with their relative contribution as depicted by respective fraction of the pie chart. Reproduced from (Mastrullo et al. 2020) article under the Creative Commons Attribution License (CC BY).

The *in vivo* bioreactor (IVB) principle refers to the harnessing of the patient's body for generating new, customized, and vascularized autologous tissue suitable for reconstructive procedures. Such strategy could be considered as a special application of the reconstructive flap prefabrication technique, thus combining the potentials of conventional reconstructive surgery and tissue engineering (Huang, Kobayashi, et al. 2016, Tan et al. 2004).

IVB has been successfully employed clinically for maxillary reconstruction by microvascular BTE flap prefabrication in a rectus abdominis muscular pouch with good manufacturing practice (GMP) level AT-MSCs, β-TCP and BMP-2, contained in a titanium cage (Mesimäki et al. 2009). The access to GMP facilities and appropriate regulatory approvals/licenses is a limiting factor which increases the cost and logistic concerns for clinical BTE approaches. Several clinical case reports have documented the application of IVB for mandibular reconstruction, with or without

intraoperatively harvested cells and BMPs, showing variable outcomes. When applied, the seeded cells were derived from intraoperatively harvested Iliac crest autografts or bone marrow aspirates (Cheng et al. 2006, Heliotis et al. 2006, Kokemueller et al. 2010, Orringer et al. 1999, Warnke et al. 2004, Warnke et al. 2006).

There is an increasing interest in BTE flap prefabrication approaches in several preclinical models aiming at clinical translation (Akar et al. 2018, Huang, Kobayashi, et al. 2016, Tatara et al. 2019). However, the predictable application of the IVB principle for BTE flap prefabrication in humans still faces many challenges. An ideal IVB strategy should minimize the use of seeded cells and growth factors. Such challenges require optimization of the local microenvironment in an adequate implantation site to improve the body self-regenerative capacity to achieve a fine balance between bone regeneration and remodeling (Heliotis et al. 2006, Huang, Kobayashi, et al. 2016, Huang, Liu, et al. 2016).

The muscular tissue can predictably serve as an IVB for BTE flap prefabrication. Both an intramuscular pouch or a pedicled flap can induce ectopic neovascularization and bone formation under suitable conditions (Ayoub et al. 2007, Huang, Kobayashi, et al. 2016, Khouri et al. 1991). Several clinical studies have adopted muscular IVB for BTE flap prefabrication (Heliotis et al. 2006, Kokemueller et al. 2010, Mesimäki et al. 2009, Warnke et al. 2004). Muscular IVB, in addition to providing a well vascularized bed, it could potentially provide skeletal progenitor cells for ectopic bone formation (Liu et al. 2014). The superiority of muscular tissue for IVB is yet to be elucidated.

BTE flap prefabrication techniques involving the periosteum are clinically promising owing to several merits. Periosteum not only can provide MSCs and important growth factors but can also create a highly neurovascular environment and simultaneous guided bone regeneration (Dimitriou et al. 2012, Huang, Kobayashi, et al. 2016). Periosteal flap IVB, however, is limited by the lack of an adequate donor site for the prefabrication of large bone grafts (Huang, Kobayashi, et al. 2016). Few studies have investigated the effect of periosteum vascularity on the quality of BTE flap prefabrication. Preclinical studies should verify the feasibility of applying periosteum based prefabricated BTE flaps in craniomaxillofacial reconstruction (Huang et al. 2017).

3 AIMS OF THE STUDY

The aim of the thesis work was to explore clinically translatable tissue engineering approaches for the management of craniomaxillofacial bone defects. This involved discovering the potential for EV-based cell-free strategies, addressing the BTE vascularization challenges, and developing a relevant translational animal model for engineering prefabricated bone flaps.

Therefore, the following objectives were set out:

- to characterize the EV-derived miRNAs and other small ncRNAs of AT-MSCs and hPSCs cultured *in vitro*, and to explore their biological relevance as key players for intercellular communication. **(Study I)**

- to investigate hypoxia-mimicking small molecules for their effects on the proangiogenic potential, proliferation, and osteogenic differentiation of AT-MSCs *in vitro.* **(Study II)**

- to compare the inherent capacity of muscle, periosteal grafts, and periosteal flaps as IVBs for prefabricating vascularized tissue engineered bone flaps with no additional cell-source or osteoinductive agents, and to assess the reconstructive potential of the prefabricated flaps in a mandibular defect preclinical sheep model. **(Study III)**

4 MATERIALS AND METHODS

4.1 Ethical permissions (Studies I-III)

An overall summary of the studies I-III is provided in Figure 4.1. In studies I and II, human AT-MSCs were isolated from donated adipose tissue samples after plastic surgery procedures. The female donors provided informed consent under the supportive statements of the ethical committee of Helsinki and Uusimaa Hospital District for the use of adipose tissue and derivatives (DNro 217/13/03/02/2015).

In study I, human pluripotent stem cells (hPSCs) which included both human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSC) were employed. The collaborators at the University of Tampere have permission of the National Authority for Medicolegal Affairs Finland (Dnro 1426/32/300/05) to use human embryos for research purposes. Supportive statements of the Ethical Committee of the Pirkanmaa Hospital District were granted (Skottman/R05116) and (Skottman/R14023).

In study III, large animal experiment was carried out according to the experiment design and surgical protocol approved by the Finnish Animal Experiment Board (ESAVI/16103/2018; 17 August 2018).

4.2 Cell culture methods

4.2.1 The culture media (Studies I and II)

The culture media which were employed in the thesis work and their compositions are summarized in Table 4.1.

4.2.2 The cells (Studies I and II)

The employed cells in the thesis work are summarized in Table 4.2.

4.2.3 Isolation and characterization of the cells (Studies I and II)

The AT-MSCs were used in studies I and II. These cells were isolated from water-assisted liposuction-aspirates donated by eight female donors after plastic procedures at the Pihlajalinna Laser Tilkka hospital, Helsinki, Finland, under the earlier ethical permission and informed consents. The fresh liposuction-aspirates underwent a combination of enzymatic and mechanical treatment for the isolation of AT-MSCs as previously described (Peltoniemi et al. 2013). For further maintenance of the plastic-adherent AT-MSCs, they were expanded in MM under standard culture conditions at 37°C and 5% CO_2 in a humidified incubator with full medium change every 3 days and passaging at 1:3 split ratio when 85% confluent. AT-MSCs from passages 2-6 were used in all experiments.

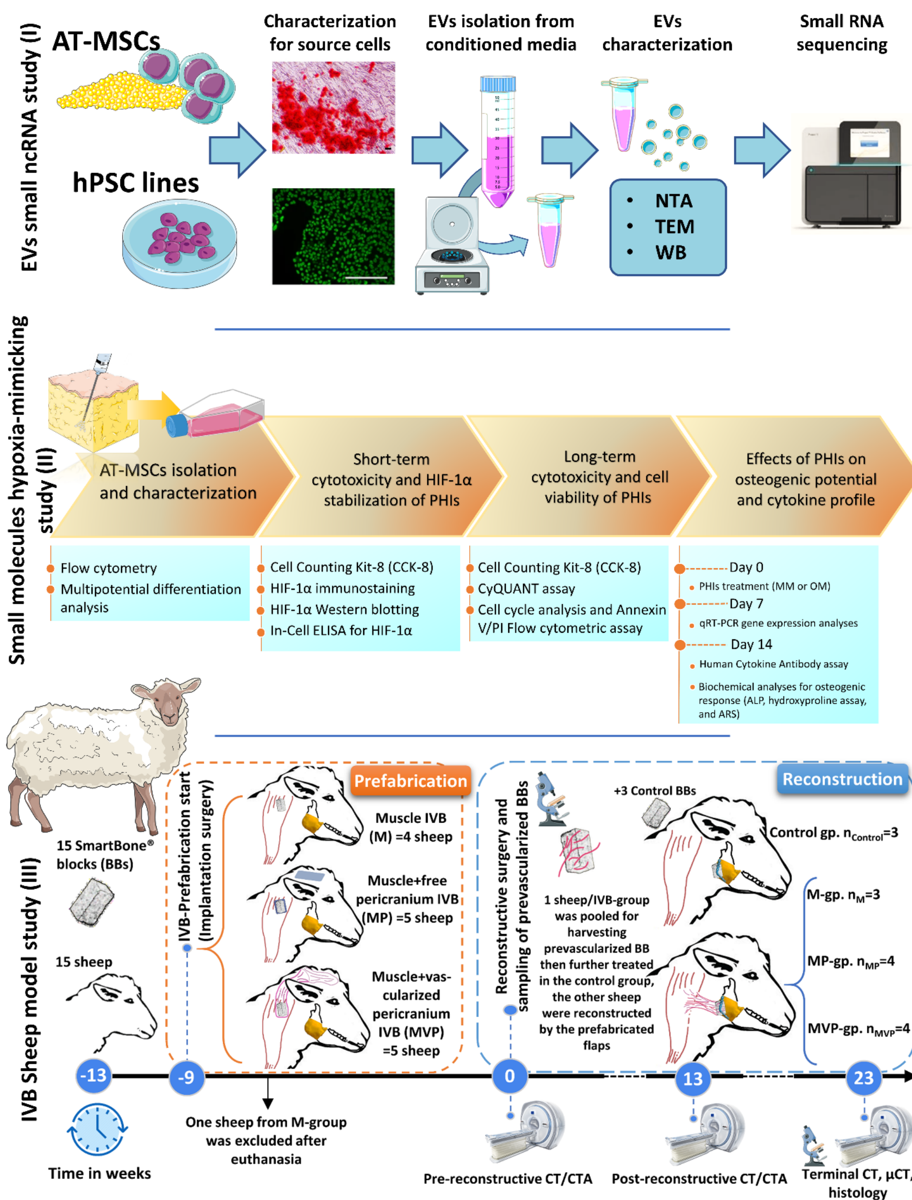

Figure 4.1: Summary of the thesis studies design.

Table 4.1: The composition of culture media employed in the thesis work

Medium	Composition	Studies
Maintenance media (MM)	Dulbecco's modified Eagle's medium/Ham's Nutrient Mixture F-12 with 1% L-alanyl-L-glutamine (DMEM/F-12 1:1 GlutaMAX; ref. 31331-028, Gibco, Grand Island, NY, USA), 1% antibiotics (100 U/mL penicillin, 0.1 mg/mL streptomycin; ref. DE17-602E, Lonza), and 10% fetal bovine serum (FBS; South American, ref. 10270-106, Gibco)	I and II
EV-depleted MM	DMEM/F-12 1:1 GlutaMAX, 1% antibiotics, and 10% EV-depleted fetal bovine serum (EV-depleted FBS; South American, ref. 10270-106, Gibco) *	I
Osteogenic media (OM)‡	MM supplemented with 50 µM L-ascorbic acid 2-phosphate, 10 mM β-glycerophosphate disodium salt hydrate, and 5 nM dexamethasone (all from Sigma-Aldrich)	I and II
Adipogenic media (AM)‡	StemPro® Adipogenesis Differentiation Kit, # A10070-01, Gibco, Thermo Fisher Scientific	II
Chondrogenic media (CM)‡	MM with reduced FBS to 1% and supplemented with 1% Insulin-Transferrin-Selenium-Ethanolamine (ITS-X, # 51500056, Gibco), 50 µg/mL L-ascorbic acid 2-phosphate (Sigma-Aldrich), 40 µg/mL L-proline (Sigma-Aldrich), 100 µg/mL sodium pyruvate (# 11360070, Gibco), 100 nM dexamethasone (Sigma-Aldrich), and 10 ng/mL of TGF-β1 (# 7754-BH-005, R&D system, Minneapolis, MN, USA)	II
Human pluripotent stem cell media (hPSC-M)	Xeno- and serum-free Essential 8™ Flex Medium (E8 flex, Thermo Fisher Scientific), supplemented with 50 U/ml Penicillin-Streptomycin (Gibco, Thermo Fisher Scientific)	I

* EV-depletion of FBS is described in Publication I
‡ Induction protocol is described in Publication II

Table 4.2: The cells used in the thesis work

Cell type	Source	Culture media	Studies
adipose tissue-derived mesenchymal stem/stromal cells (AT-MSCs)	human adipose tissue and liposuction aspirates	MM, EV-depleted MM, OM, CM, AM	I and II
human embryonic stem cells (hESCs)	blastocyst stage human embryo[1]	hPSC-M	I
human induced pluripotent stem cells (hiPSC)	dermal fibroblasts[2]	hPSC-M	I

[1] Regea-Institute for Regenerative Medicine, University of Tampere and Tampere University Hospital, Finland
[2] Prof. Katriina Aalto-Setälä's laboratory, University of Tampere, Finland

The characterization of AT-MSCs involved the assessment of surface antigens of interest based on the ISCT-position statement (Dominici et al. 2006). The flow cytometry analysis utilized a BD Accuri C6 flow cytometer (Becton–Dickinson, Franklin Lakes, NJ, USA) and allophycocyanin (APC)-conjugated monoclonal antibodies against CD14 (clone: M5E2), CD19 (clone: HIB19), CD34 (clone: 581), CD45RO (clone: UCHL1), CD54 (clone: HA58), CD73 (clone: AD2), CD90 (clone: 5E10), CD105 (clone: 266), and HLA-DR (clone: G46-6) (BD Pharmingen, Becton-Dickinson, Franklin Lakes, NJ, USA). For that, AT-MSCs were expanded in MM up to passage 5 and 1×10^4 events were recorded per sample. For each sample, the level of fluorescence was measured above that of the corresponding unstained cell sample. Additionally, multipotentiality of AT-MSCs was assessed by analyzing their capacity to differentiate toward the adipogenic, osteogenic, and chondrogenic lineages under the corresponding differentiation media for 3 weeks.

Two hPSC lines were used in study I (hESC line hPSC-1 and hiPSC line hPSC-2). Both hPSC lines were cultured and characterized at the Faculty of Medicine and Life Sciences, BioMediTech, University of Tampere, Tampere, Finland, under their standard protocols which are described in detail in Publication I. The provided conditioned and unconditioned hPSC-M media were used in study I.

4.2.4 Prolyl-hydroxylase inhibitors (PHIs) (Study II)

The chemical induction of a hypoxic response in AT-MSCs involved the use of two small-molecule drugs; DMOG (D3695, CAS: 89464-63-1, lot # 086M4731V) and baicalein (465119, CAS: 491-67-8, lot # MKBV1595V), both from Sigma-Aldrich (St. Louis, MO, USA). DMOG and baicalein were dissolved in DMSO (MP Biomedicals, LLC, Illkirch Cedex, France). DMSO was used as a negative control and $CoCl_2 \cdot 6H_2O$ (Merck,

Darmstadt, Germany. Art.2539) served as a positive control for comparison of treatment effects. AT-MSCs were assessed for their osteogenic differentiation after 14 days of OM culture with or without the tested PHIs at the optimized concentrations (3.75 pmol/cell of DMOG and 0.25 pmol/cell for baicalein) based on cellular viability response.

4.3 EV isolation and basic characterization (Study I)

For hPSCs-M conditioned media, cell debris were removed by centrifugation on 2000*g* for 10 min at +4°C. The supernatant was stored in new Falcon tubes at -80°C. The AT-MSCs-conditioned EV-depleted MM were cleared of cell debris by centrifugation at 2500*g* for 10 min, followed by filtering the supernatant through a 0.45 µm sterile filter (Merck Millipore).

The EVs were extracted using ultracentrifugation at 26,000 rpm (121 896 gmax) for 2 hours at +4°C with SW28 rotor (k-factor 284.7, Beckmann-Coulter). The EV pellet was washed by filtered PBS and stored in Protein LoBind microcentrifuge tubes (Eppendorf) at -80°C. The EVs for RNA sequencing were isolated from the conditioned medium by precipitation using the miRCURY™ Exosome Isolation Kit (Exiqon A/S, Vedbaek, Denmark) according to the manufacturer's instructions. The isolated EV samples underwent characterization by the nanoparticle tracking analysis (NTA), transmission electron microscopy (TEM), and western blotting. Detailed description for the NTA and TEM methods are included in Publication I. Relevant data of the EV characterization was submitted to the EV-TRACK knowledgebase (EV-TRACK ID: EV180022).

4.4 EV-RNA isolation and small RNA sequencing (Study I)

During the EV isolation by miRCURY™ Exosome Isolation Kit (Exiqon A/S) before precipitation, cellular components and unprecipitated material were removed by two centrifugation and supernatant removal steps. Subsequently, EVs were lysed and the RNA was isolated by miRCURY™ RNA Isolation Kits - Cell & Plant (Exiqon A/S). The sequencing experiments for two hPSC-EV samples, three AT-MSC-EV samples, and their corresponding unconditioned media samples were performed at Exiqon Services, Denmark. More details are found in Publication I and the RNA sequencing data has been deposited to the GEO (accession number GSE113868).

4.5 Biochemical analyses for cellular viability (Study II)

AT-MSCs were cultured on 96-well plates at 1.6×10^3 cells/well in 100 µL of MM and allowed to attach for 24 h in a humidified incubator. Then, the cells received different concentrations of tested PHIs in MM or the control conditions (details in Publication II). The viability of the AT-MSCs at different timepoints after short-term and long-term (up to 14 days) PHIs treatment was assessed by Cell Counting Kit-8 (CCK-8) (# CK04-11, Dojindo Molecular Technologies, Rockville, MD, Maryland, USA) according to the manufacturer instructions.

The CyQUANT™ cell proliferation assay kit was used to estimate the total cellular DNA in cell lysates according to the manufacturer instructions. AT-MSCs were lysed in 0.1% Triton-X 100 (Sigma-Aldrich) after PHIs treatment for up to 14 days. The resulting Fluorescence was measured with VICTOR™ microplate reader at 480/520 nm excitation/emission maxima. The results of the viability assays were used to estimate the optimized concentrations for long-term treatment by the tested PHIs based on a dose-viability curves.

4.6 Flow cytometry for cell cycle analysis and Annexin V/PI (Study II)

The selected PHIs concentrations were assessed for their effects on the AT-MSCs cell cycle and apoptosis after 4 and 14 days of PHIs treatment. The cell cycle analysis involved the fixation of harvested cells in 70% ethanol, the washed fixed cells were stained with FxCycle™ PI/RNase Staining Solution (# F10797, Thermo Fisher Scientific) according to the manufacturer's protocol, then underwent flow cytometry using BD Accuri C6 flow cytometer with 488 nm excitation and collected emissions by a 585/40 filter.

In a parallel setting, harvested PHIs-treated AT-MSCs were washed and stained using Dead Cell Apoptosis Kit with Annexin V Alexa Fluor™ 488 and Propidium Iodide (PI) (# V13241, Thermo Fisher Scientific) according to the manufacturer's protocol. Subsequent flow cytometric analysis using BD Accuri C6 involved the 488 nm excitation and collected emissions with 530/30 and 585/40 bandpass filters.

4.7 Immunofluorescent detection of HIF-1α (Study II)

After treatment with PHIs or control conditions for 5 h, AT-MSCs on coverslips were fixed in 4% paraformaldehyde (PFA) for 10 min and rinsed 3 × 5 min in PBS followed by permeabilization using 0.5% Triton X-100 for 20 min and washing in PBS. After blocking in 10% normal donkey serum for 1 h at RT, cells were incubated overnight with primary mouse anti-human HIF-1α (# 610959, BD Biosciences, Franklin Lakes, NJ, USA) at a dilution of 1:50 in 0.5% normal donkey serum at 4°C. After rinsing, the coverslips were incubated with both donkey anti-Mouse IgG secondary antibody (Alexa Fluor 568, # A10037, Life Technologies, Eugene, OR, USA) in 5 μg/ml dilution and CellTrace™ 1:1000 (CFSE Cell Proliferation Kit, # C34554, Life technologies) for 1 h at RT. Cells nuclei were stained in Hoechst 33342 (# B2261, Sigma-Aldrich) for 30 min in the dark, followed by washing and mounting on a glass slide with SlowFade® mountant (# S36967, Thermo Fisher Scientific, Waltham, MA, USA). The cells were imaged on a Leica TCS SP8 confocal microscope (Leica Microsystems GmbH, Wetzlar, Germany).

The In-Cell ELISA Near Infrared Detection Kit (# 62201, Thermo Fisher Scientific) was used according to the manufacturer instructions for assessing the HIF-1α stabilization response with different DMOG and baicalein concentrations. AT-MSCs, treated in black 96-well clear-bottom plates, were fixed by 4% methanol-free formaldehyde (# 28906, Thermo Scientific), followed by washing, permeabilization, and blocking

steps. HIF-1α was probed using mouse anti-human HIF-1α (1:50, # 610959, BD Biosciences). A housekeeping antibody of rabbit polyclonal beta actin (2 µg/mL, # PA5-16914, Thermo Fisher Scientific) was used. Primary antibodies were incubated overnight at +4°C. AT-MSCs were washed (1 × wash buffer) before incubation with species-specific near-infrared DyLight-conjugated secondary antibody mix for 1 h in RT. The Odyssey FC Imager (LI-COR) served to scan the plates with excitation/emission maxima of 692/712 nm for DyLight 680 Dye and 777/794 nm for DyLight 800 Dye. The measured signals were analyzed with Image Studio Software (LI-COR).

4.8 Western blotting (WB) (Studies I and II)

Western blotting was performed for the characterization of the EV samples (Study I) and for assessing HIF-1α and VEGF in AT-MSCs lysates after PHIs treatment for 5 h (Study II). In study II, 22 µg of protein/sample were used as measured by Pierce™ BCA Protein Assay Kit (# 23227, Thermo Fisher Scientific). Generally, samples were denatured at 95°C for 5 min in reducing Laemmli sample buffer except for CD63 detection which was run in non-reducing conditions. Proteins were separated using Mini-PROTEAN® TGX™ 12% gradient SDS-PAGE gel (Bio-Rad, Hercules, CA, USA) with prestained protein ladder (BlueSTAR Prestained Protein Marker, # MWP03, Nippon Genetics Europe GmbH) as a standard. The running conditions were 150 V for 60 minutes. Blotting involved semi-dry transfer of proteins on nitrocellulose membranes 0.2 µm (#162–0112, BIORAD) using 40 mA per gel for 60 minutes. Blocking and primary antibody (Table 4.3) incubations were performed in Odyssey blocking buffer (LI-COR), for antibody incubations 0.1% Tween-20 was added. After primary antibody overnight incubation at +4°C, membranes were washed 4 × 5 minutes in TBS-T, and probed with secondary IRDye® 800CW Goat (Li-COR) at 1: 15,000 for 1 hour at RT. After incubation, membranes were washed 4 × 5 minutes in TBS-T at RT and briefly rinsed with PBS 1×, then imaged on an Odyssey FC Imager (Li-COR). Whenever applicable, the normalization of the target signal was achieved by REVERT™ Total Protein Stain kit (# 926-11010, LI-COR, Lincoln, NE, USA) according to manufacturer's instructions.

4.9 Biochemical analyses for AT-MSCs osteogenic potential (Studies I and II)

The osteogenically induced AT-MSCs, with or without PHIs, were lysed using 0.1% triton-x-100 and freezing at -80°C for subsequent alkaline phosphatase (ALP) assay or hydroxyproline assay. ALP activity was measured by mixing the cell lysate with *p*-nitrophenyl phosphate disodium (# P5744, Sigma-Aldrich) and 2-amino-2-methyl-1-propanol (# A9226, Sigma-Aldrich). The product *p*-nitrophenol absorbance was measured in a VICTOR™ microplate reader at 405 nm.

Table 4.3: The unconjugated primary antibodies used in the thesis work

Antibody	Host, clonality	Catalog, manufacturer	Dilution/conc.	Method
Study I				
Hsp70	Mouse, m	554243, BD Biosciences	1:1000	WB
CD63	Mouse, m	556019, BD Biosciences	1:1000	WB
TSG101	Mouse, m	SAB2702167, Sigma-Aldrich	1:500	WB
CD90	Mouse, m	WH0007070M1, Sigma-Aldrich	1:500	WB
Calnexin (C5C9)	Rabbit, m	2679, Cell Signaling Technology	1:800	WB
Study II				
HIF-1α	Mouse, m	610959, BD Biosciences	1:50, 1:500	IF, WB*
VEGF (C-1)	Mouse, m	sc-7269, Santa Cruz	1:200	WB
beta Actin	Rabbit, p	PA5-16914, Thermo Fisher Sc.	2 µg/mL	IF
Study III				
Von Willebrand Factor	Rabbit, p	ab6994, Abcam	1:1000	IHC-P
m=monoclonal, p=polyclonal, *respectively				

The hydroxyproline assay Kit (# MAK008, Sigma-Aldrich) was used according to the manufacturer protocol for quantifying the hydroxyproline concentration in cell lysates which is correlated to the collagen content. The cell lysates were hydrolyzed in 6 N hydrochloric acid at 120°C for 3 h, oxidized hydroxyproline reacted with 4-(dimethylamino) benzaldehyde for 90 min at 60°C. Absorbance of the colorimetric product was measured at 550 nm in VICTOR™ microplate reader. Normalization of the ALP activity and total collagen content to cell number was achieved by quantifying the amount of DNA in the cell lysates by the CyQUANT™ cell proliferation assay kit.

The quantification for the ECM mineralization was assessed by Alizarin Red S Staining (ARS). AT-MSCs were fixed in ice-cold 70% ethanol for 1 h at RT, followed by rinsing twice with ddH_2O. The Cells were stained with 2% ARS solution (# A5533-25G, Sigma-Aldrich) for 30 min at RT, followed by thorough washing for 5 × 5 min in fresh ddH_2O under shaking. The Bound ARS was extracted using 100 mM N-cetylpyridinium chloride monohydrate (Merck) in ddH_2O for 2 h in 37°C followed by measuring the eluted stain at 550 nm in the microplate reader. For cell number normalization, the fixed cells were stained by 2 mM Janus Green B Stain (# 201677-25G, Sigma-Aldrich) for 5 min at RT. After washing for 5 × 5 min in fresh ddH_2O, 0.1 mL 0.5 M HCL was added per well and left for 10 min to elute the stain. After shaking the plate for 10 s, the absorbance was measured at 550 nm in VICTOR™ microplate reader.

4.10 Human cytokine antibody assay for AT-MSCs conditioned media (Study II)

After treating AT-MSCs with MM or OM with or without PHIs for 14 days, the cells conditioned a serum-free media (DMEM/F-12 1:1 GlutaMAX with 1% antibiotics (100 U/mL penicillin, 0.1 mg/mL streptomycin) for 24 h. The conditioned medium was collected and centrifuged (1000*g* for 10 min, +4°C). The supernatant was stored in -80°C until further analysis. The analysis of the secreted cytokines was performed using human cytokine antibody array membranes (# ab133998, Abcam, Cambridge, UK) following the manufacturer's protocol. The signal detection was performed using ChemiDoc XRS Imaging System (Bio-Rad). The ImageJ software (National Institutes of Health) was used to quantify the intensity of individual dots by densitometric analysis. The normalized signal density of each dot was calculated, MM with DMSO served as a reference. The background error was estimated by taking the difference between the average of negative controls and the lowest value on the assay. The final analysis included only the cytokines which were detected above experimental error in at least one sample.

4.11 Real-Time qRT-PCR (Study II)

After treating AT-MSCs for 1 week with MM or OM with or without PHIs, we analyzed their gene expression for selected genes of interest related to osteogenesis and stemness-related genes. The RNA isolation was performed using the miRCURY™ RNA Isolation Kit (Exiqon A/S) according to the manufacturer's instructions. Reverse transcription into cDNA was done using a SuperScript™ IV VILO™ reaction mixture (# 11766050, Thermo Fisher Scientific). PCR reactions were conducted on a QuantStudio™ 5 Real-Time PCR System (Thermo Fisher Scientific) or Rotor-Gene Q (Qiagen, Hilden, Germany) with TaqMan™ Universal Master Mix II (# 4440038, Thermo Fisher Scientific) or 5 × HOT FIREPol EvaGreen qPCR Mix Plus (no ROX) (# 08-25-00001, Solis BioDyne, Tartu, Estonia). The details for the used TaqMan® assays (Thermo Fisher Scientific) and primers are found in Table 4.4. After normalizing the signals with housekeeping genes, the data was analyzed using the $2^{-\Delta\Delta Ct}$ method to quantify relative gene expression.

4.12 The ovine IVB model and surgical plan (Study III)

The sheep were purchased from a licensed commercial vendor and housed in group pens under the standard housing conditions in the large animal facility of the Laboratory Animal Centre (LAC) of the University of Helsinki. They comprised 15 skeletally mature female Texel and Crossbred sheep (24-35 months; 51-65 kg (56.4 ± 4.3 kg)). The sheep were allowed to acclimatize for 4 weeks before any intervention.

Table 4.4: The TaqMan® assays and primers used in study II

TaqMan® assays (Thermo Fisher Scientific)		
Genes	Symbol	Assay ID
Alkaline phosphatase, liver/bone/kidney	*ALPL*	Hs01029144_m1
Bone morphogenetic protein 2	*BMP2*	Hs00154192_m1
Runt-related transcription factor 2	*RUNX2*	Hs01047973_m1
Vascular endothelial growth factor A	*VEGFA*	Hs00900055_m1
Secreted phosphoprotein 1	*SPP1*	Hs00959010_m1
Collagen type I alpha 1	*COL1A1*	Hs00164004_m1
Ribosomal protein lateral stalk subunit P0	*RPLPO*	Hs99999902_m1
Sclerostin	*SOST*	Hs00228830_m1

The primers used for stemness-related genes*			
Genes	Primer sequences	Size	origin
Kruppel-like factor 4 (*KLF4*)	Forward: 5'-CCGCTCCATTACCAAG-3' Reverse: 5'-CACGATCGTCTTCCCCTCTT-3'	80 bp	hum (NM_004235.4)
Nanog homeobox (*NANOG*)	Forward: 5'-CTCAGCCTCCAGCAGATGC-3' Reverse: 5'-TAGATTTCATTCTCTGGTTCTGG-3'	94 bp	hum (NM_024865.2)
Octamer-binding transcription factor 4 (*OCT4*)	Forward: 5'-TTGGGCTCGAGAAGGATGTG-3' Reverse: 5'-TCCTCTCGTTGTGCATAGTCG-3'	91 bp	hum (NM_002701)
Cyclophilin G (*CycloG*)	Forward: 5'-TCTTGTCAATGGCCAACAGAG-3' Reverse: 5'-GCCCATCTAAATGAGGAGTTG-3'	84 bp	hum (NM_004792)

*The primers were a kind gift from Professor Otonkoski lab

The sheep underwent two surgical interventions under general anesthesia (GA) (Figure 4.1; study III). In the first surgery, cell-free alloplastic bone blocks were implanted with three IVB techniques (one block per sheep/IVB). In this surgery, sheep were randomly allocated as 5 sheep per tested IVB. The tested IVBs were an intramuscular pouch (M) in the rostral part of brachiocephalic muscle; a pericranial/periosteal graft with the muscular pouch (MP); and a pericranial/periosteal vascularized flap into the same muscular pouch (MVP). After a *prefabrication period* of 8-11 weeks, the sheep underwent the second surgery. The prefabricated tissue engineered bone (TEB) flap was raised as an island pedicled flap and was used for reconstructing a critical-sized defect (CSD) of the ipsilateral mandibular angle. In three sheep (one per each IVB technique), the prefabricated TEB blocks were harvested for histological evaluation, the CSDs were reconstructed using non-prevascularized bone blocks as a control. One sheep from M-group suffered from postoperative cardiopulmonary complications after the first surgery, it therefore underwent euthanasia based on the veterinarian decision and was

excluded from the study. The later assessment of the reconstructive phase comprised 14 sheep (n=14) in four groups: the control group ($n_{Control}$=3); M-group (n_M=3); MP-group (n_{MP}=4); and MVP-group (n_{MVP}=4). The overview for the experimental groups, postoperative analyses, and the study III timeline is presented in Figure 4.1. The details of the GA and perioperative management are provided in Publication III.

4.13 The surgical procedures (Study III)

4.13.1 First stage surgery (prefabrication in IVBs phase)

All the surgical procedures were performed in the operating theatres of the Equine and Small Animal Medicine, Veterinary Teaching Hospital, University of Helsinki. Food was withheld for at least 12 hours prior to any intervention under GA, with water accessible ad libitum. Under GA, with the sheep put to a left lateral recumbency position, the fleece over the right aspect of the neck and forehead was trimmed and the skin of the surgical field was carefully disinfected, prepped, and draped in a sterile fashion. Prior to incision, the tissues were infiltrated with local anesthetic (LA) lidocaine cum adrenaline (maximum 4 mg/kg of lidocaine). A lazy S incision was carried out on the dorsal right aspect of the neck, a rostral extension of the incision was performed over the forehead when the exposure of the pericranium was needed in MP and MVP groups (Figure 4.2; D). Sharp and blunt dissection with meticulous hemostasis was performed through subcutaneous tissues followed by the creation of the brachiocephalic muscular pouch (Figure 4.2; A and B) at its rostral part ventral to the splenius muscle. The bone blocks (BBs) were implanted into the muscular pouches in M-group sheep after soaking in venous blood (Figure 4.2; C). In MP-group sheep, a nonvascularized pericranial graft was harvested for wrapping the blood-soaked BB with the cambium layer facing inwards (Figure 4.2; D-I). In MVP-group, an axial pericranial vascularized flap based on branches of the occipital, posterior auricular, and posterior meningeal arteries was raised (Figure 4.2; J). The blood-soaked BB was wrapped with the vascularized pericranial flap facing its cambium layer. In both MP- and MVP-group, the pericranium-wrapped BBs were implanted in a similar muscular pouch as done in M-group (Figure 4.2; I and K). After infiltrating tissues with 5 ml long acting LA (Ropivacain, 10 mg/ml, Fresenius Kabi AB), the muscular pouch and subcutaneous tissues were closed by resorbable Vicryl 3-0 suture (Ethicon), and the skin was closed by 2-0 Ethilon suture (Figure 4.2; L) to be removed 10 days postoperatively.

4.13.2 Second stage surgery (reconstructive phase)

After the pre-reconstructive CT scans, the sheep were immediately moved to the operating room where they were prepared for the second surgery under GA by positioning, prepping, and draping in a sterile fashion, as previously described, exposing the right dorsolateral aspect of the neck and the right submandibular region. Similar to the first surgery, the same protocol for LA infiltration was applied.

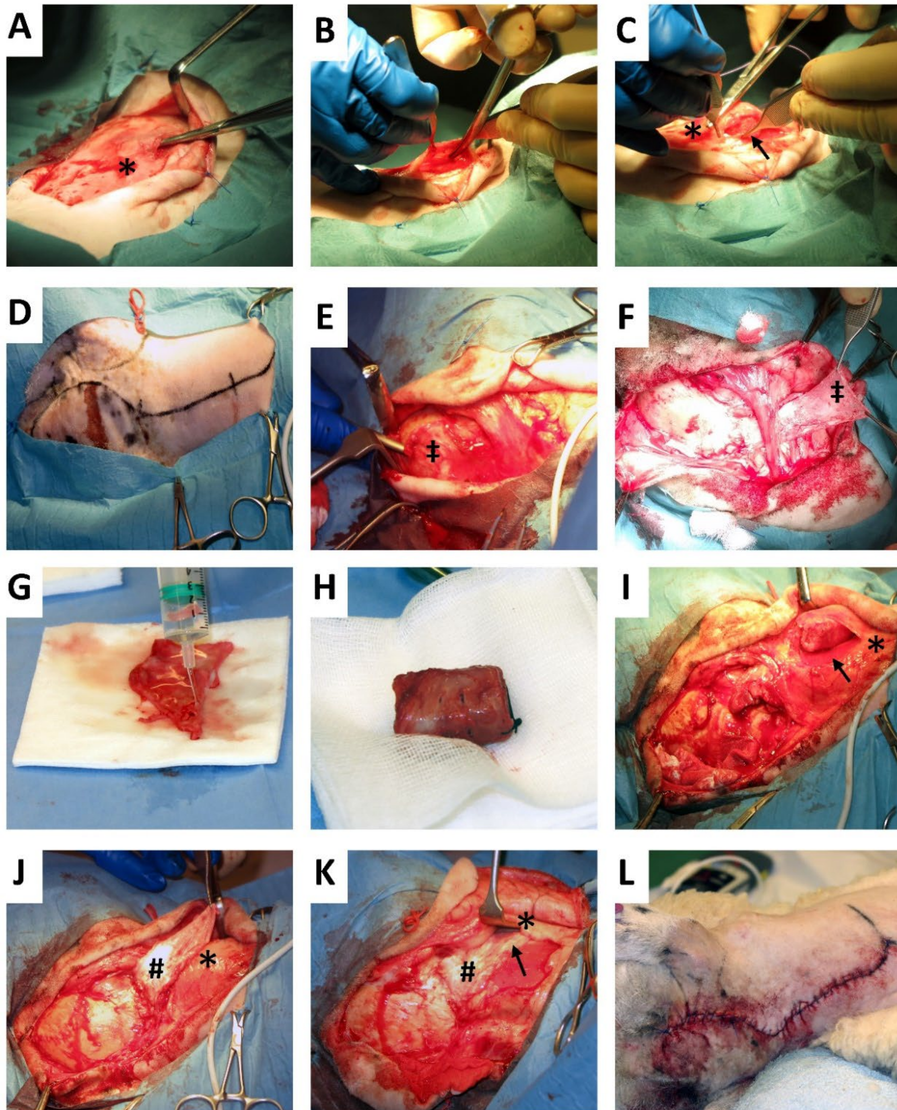

Figure 4.2: The first surgery for implanting the bone blocks (BBs) into the tested IVBs, i.e., muscle pouch (M) (A-C), pericranial graft with muscle pouch (MP) (D-I), or pericranial flap with muscle pouch (MVP) (J-L). In all the sheep, the muscular pouch (arrow) was created in the brachiocephalic muscle (*) (A and B). In M-group, the BBs were inserted directly into the pouch (C). In MP-group, a periosteal/pericranial graft (‡) was elevated by a periosteal elevator (E), harvested (F and G) to wrap the BBs (H) before implanting in the muscular pouches (arrow) (I). In MVP-group, periosteal/pericranial vascularized flaps (#) were elevated to wrap the BBs before implanting in the muscular pouches (J and K). The surgical wound was closed in layers (L).

A lazy S incision was performed on the right lateral aspect of the neck with a submandibular extension towards the right mandibular angle region. Sharp and blunt dissection with careful hemostasis was performed to expose superficial neck muscles. The prefabricated TEB flap was raised by sharp dissection through the brachiocephalic muscle to include the prevascularized BB with a surrounding muscle tissue as a myoosseous flap (Figure 4.3). The flap dissection involved the preservation of the pedicle which comprises occipital artery branches to the muscle segment (Figure 4.3; A and B). These branches showed a consistent close relation to the accessory nerve. The right mandibular angle was exposed through the sharp dissection of the pterygomasseteric sling. A CSD corresponding to the intraoperative dimensions of the BB in the prefabricated TEB flap was created using a bone saw (Stryker or DePuy Synthes) under saline irrigation. The defects were 29 (±2) × 18 (±1) mm in average. The transplanted flap was used for CSD reconstruction after careful exposure of the BB surfaces which faced the CSD edges (Figure 4.3; C-E). The internal fixation was accomplished by a miniplate and screws (Figure 4.3; E). In three randomly assigned sheep (one sheep/IVB), the prevascularized BBs were harvested for histological analysis and the CSDs were reconstructed using fresh blood-soaked BB, and these constituted the control group. The surgical wound was closed in layers with resorbable 3-0 Vicryl and PDS-II (Ethicon).

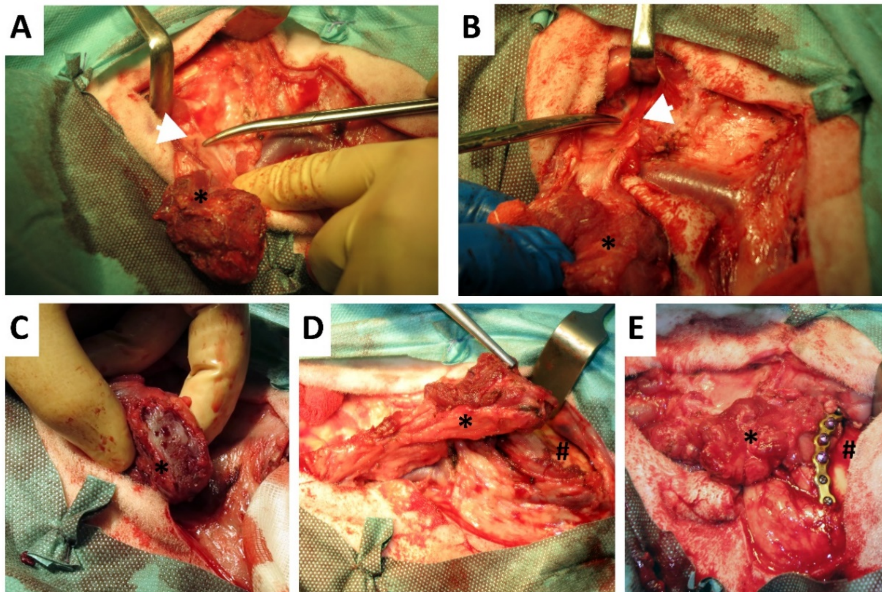

Figure 4.3: The second (reconstructive) surgical phase. The instrument and white arrow heads show the vascular pedicle during raising the prefabricated TEB flap (✱) (A and B). Evident vascularization through the biomaterial pores was seen intraoperatively (C). The prefabricated TEB flap (✱) was transplanted for reconstruction of mandibular angle (#) bone defect (D and E).

4.14 The stock bone blocks (BBs) (Study III)

Commercially available SmartBone® blocks (15×30×20 mm) (#NFHU011210, Industrie Biomediche Insubri S/A, Mezzovico-Vira, Switzerland) were purchased. The constituent biomaterial is biohybrid in nature, consisting of bovine-derived mineral matrix which is reinforced with resorbable poly(lactic-co-caprolactone) copolymer and RGD-exposing collagen fragments for surface activation. SmartBone® has been previously characterized *in vitro*, *in vivo,* as well as in clinical trials (Ferracini et al. 2019, Pertici et al. 2015, Sallent et al. 2020).

4.15 Computed tomography (CT) and micro-CT (µCT) analysis (Study III)

Sheep underwent CT-scans for their heads in a LightSpeed VCT 64 slice CT Scanner (GE Medical Systems, USA) at the specified timepoints on the study outline (Figure 4.1). All CT-scans were performed under GA, except for the terminal endpoint CT which was performed immediately after euthanasia. CT angiography (CTA) was performed for their head and neck region after the prefabrication phase and at the post-reconstructive follow up. The details for the GA, scanning parameters, and analysis of CT data are provided in Publication III.

The µCT analysis was performed on the explanted samples of the reconstructed mandibular defects after euthanasia at 23 weeks post-reconstruction. The µCT-scans were performed with a GE phoenix nanotom s system (General Electric Sensing and Inspection Technologies/Phoenix X-ray, Germany) at the University of Helsinki X-Ray Micro-CT Laboratory. The details for scanning parameters and µCT data analysis are provided in Publication III.

4.16 Histological analysis (Study III)

The studied samples included the explanted prevascularized bone blocks after the prefabrication phase in sheep (one block from each IVB group) and the mandibular samples after the terminal µCT (Figure 4.1). The samples were immersion-fixed in 10% neutral buffered formalin (NBF) for 12 days and sectioned into smaller segments to allow the analysis of different parts of the reconstructed defect. Samples were decalcified in 0.5 M Ethylenediaminetetraacetic acid (EDTA) 7.5 pH for 12 weeks, routinely processed, embedded in paraffin, and sectioned at 4 µm thickness. The decalcified sections were stained with hematoxylin and eosin (H&E) and Masson's trichrome (MTC). Selected sections were stained with picrosirius red, reticulin, and Movat's pentachrome staining. Mid-defect samples from each animal were processed as undecalcified sections by BioSiteHisto (BioSiteHisto Oy, Tampere, Finland). The formalin-fixed samples were dehydrated in ascending alcohol series, cleared in xylene, and embedded gradually into methyl methacrylate (MMA). The 5µm-thin slices were sectioned with a hard tissue microtome (Leica, SM2500 Large Scale, Heavy duty Sectioning System) and collected on albumin-glycerin coated slides. The slides were heated at +60°C for 3-5 days after sectioning for better adherence of the sections to the slides. The sections were then stained by Masson

Goldner Trichrome (MT) stain. For the subsequent analyses and measurements, the slides were digitalized as a whole-slide image (WSI) with Pannoramic 250 FLASH II, 3DHISTECH (3DHISTECH, Budapest, Hungary) with a 20× air objective, the images were saved in MRXS-files. The WSIs were viewed and analyzed using CaseViewer version 2.4 (3DHISTECH, Budapest, Hungary).

Immunohistochemical (IHC-P) staining was performed on prevascularized BB samples for assessing the vascularization. After heat-induced antigen retrieval (20 minutes at 99°C in 10 mM citrate buffer, pH 6), the IHC-P utilized the anti-von Willebrand factor (vWF) antibody (1:1000; rabbit polyclonal, Ab6994, Cambridge, UK) (Table 4.3), detected with polymer-linked secondary antibody and peroxidase (BrightVision + Poly-HRP kit, ImmunoLogic, Duiven, Netherlands) and DAB chromogen according to the manufacturer's instructions. Subsequent analysis was performed using the open-source digital pathology software QuPath version 0.2.3 and CaseViewer version 2.4 (Publication III).

4.17 Statistical analysis

The results are presented as means ± standard deviations (SD) or standard error of mean (SE). The averaged technical replicates for each biological replicate (n) were analyzed in OriginPro (2020-SR1-9.7.0.188) or earlier versions (OriginLab Corporation, Northampton, MA, USA). The paired samples *t*-test was applied to assess differences between the two timepoints. Analysis of variance (ANOVA) was employed; either one-way or two-way depending on independent variables followed by Bonferroni-corrected post hoc tests to analyze specific sample pairs for significant differences. Equality of variances were pre-assessed by Levene's test. Statistical significance was set at p-values < 0.05.

5 RESULTS

5.1 Characterization of AT-MSCs (Studies I and II)

The human donors for adipose tissue had an age range of 32-53 years with a mean (±SD) of 42.7 (±8.2) years and a body mass index (BMI) range of 22.7-31.2 with a mean of 26 (±3). The isolated AT-MSCs showed the characteristic morphology, surface marker profile, and differentiation potential as suggested by the ISCT (Dominici et al. 2006). The plastic-adherent fibroblast-like cells highly expressed CD73, CD90, and CD105. They lacked the expression of hematopoietic markers CD14, CD19, CD45, and HLA-DR. The isolated cells showed moderate expression of CD54 and CD34 with evident donor variability (Table 5.1). The AT-MSCs showed differentiation potential towards adipogenic, osteogenic, and chondrogenic lineages under the respective induction media (Figure 5.1).

Table 5.1: Surface marker expression (%) of the employed undifferentiated AT-MSCs as measured by flow cytometry

Surface protein	**Antigen**	**mean (±SD) n=8**	**Expression**
Serum lipopolysaccharide-binding protein	CD14	0.2(±0.14)	-
B-lymphocyte antigen	CD19	0.1(±0.08)	-
Sialomucin-like adhesion molecule	CD34	26.5(±16.73)	+
Leukocyte common antigen	CD45	0.2(±0.14)	-
Inter-Cellular Adhesion Molecule	CD54	53.9(±27.13)	+
ecto-5'-nucleotidase	CD73	99.5(±1.12)	++
Thy-1 (T cell surface glycoprotein)	CD90	99.8(±0.25)	++
SH-2, endoglin	CD105	97.7(±2.3)	++
Major histocompatibility class II antigens	HLA-DR	0.4(±0.38)	-

5.2 EVs characterization (Study I)

EVs were isolated by ultracentrifugation from the conditioned media of AT-MSCs and hPSCs. The detection of EVs by TEM revealed the presence of various intact EVs of different sizes and electron densities in all the samples (Publication I; Figure 3-D and E). The NTA results showed that most of the detected particles were in the range of 100-200 nm. However, larger particles (201-300 nm) were more abundant in AT-MSCs-conditioned media. Generally, more particles were detected in hPSCs-conditioned media (1.8-2.3 × 10^{11} particles/ml) as compared with AT-MSCs-conditioned media (2-4 × 10^{10} particles/ml) (Publication I; Figure 3-A and B). The unconditioned hPSCs-M media contained negligible amounts of EV-sized particles, while the EV-depleted FBS which showed large number of particles (100-200 nm). However, those particles are mostly protein aggregates and lipoproteins, as confirmed with TEM and WB results.

EV markers were analyzed for TSG101, Hsp70, CD63, and CD90 by WB (Table 4.3) (Publication I; Figure 3-C). The unconditioned media did not show positive signal towards any of the probed markers. The endoplasmic reticulum marker calnexin was not detected in any of the EV samples. TSG101 was detected in all EV samples, Hsp70 was also detected in hPSCs-EVs. CD63 and CD90 were detected in all EV samples with a relatively stronger signal in AT-MSCs-EVs (Publication I; Figure 3-C).

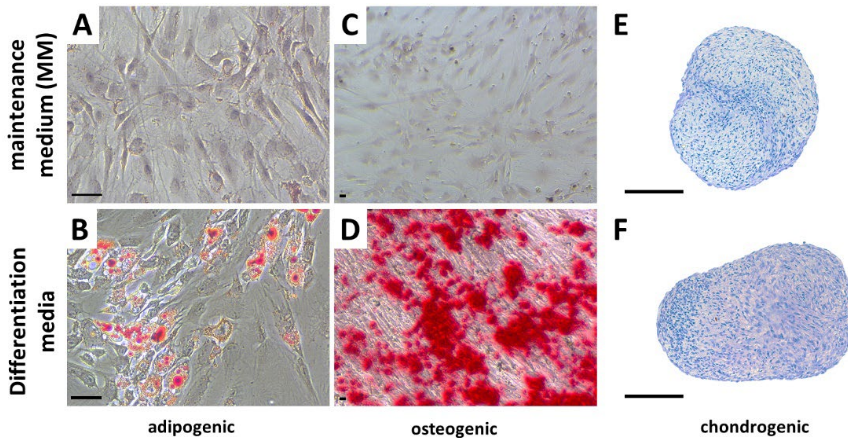

Figure 5.1: Photomicrographs show multipotentiality of tested AT-MSCs. Cells were induced for 3 weeks with AM which resulted into the gradual accumulation of intracellular lipid droplets as detected by Oil Red O staining (B). Treatment with OM for same duration enhanced the mineralized matrix formation as detected by Alizarin Red S staining (D). Chondrogenic potential was verified by treating AT-MSCs for 3 weeks with CM, extracellular glycosaminoglycans were stained by Toluidine blue metachromatic staining (F). MM (A, C, and E) was used in parallel as a negative control for differentiation conditions. Scale bar = 200 µm

5.3 The miRNA signature for hPSCs and AT-MSCs

Next generation sequencing was employed for determining the small ncRNA expression profile for EV samples from hPSCs (2 samples) and AT-MSCs (3 samples) and the corresponding unconditioned media. The reads obtained from the unconditioned media, which were relatively higher with the EV-depleted MM of AT-MSCs, were considered as a background for the subsequent analyses. Briefly, the raw read counts were normalized to external spike-ins (UniSp100-UniSp151), the analyses included only RNAs which had at least 2×fold higher spike-in-normalized counts in the EV samples (|Fold change| > 2) as compared to those in the corresponding media. Meanwhile, RNAs with similar expression levels between samples (|Fold change| < 2) were required to have at least 2-fold normalized counts in both sample groups.

The unsupervised cluster analysis of EV-miRNAs (Figure 5.2; A) and -ncRNAs (Figure 5.2; B) was performed. EVs from hPSC and AT-MSCs showed clearly distinct

expression patterns, however, a strong similarity within each group was evident (Figure 5.2). EV-miRNAs were divided into groups based on significant (*p*-value < 0.01) differential expression or expression in both sample types ($|FC| < 2$) and a cutoff of 32 mean counts per million (CPM). AT-MSCs related EV-miRNA exclusively comprised 32 miRNAs which are positive and negative regulators of osteogenic differentiation, they included e.g., mir-families: mir-10/100, mir-125, mir-196, mir-199, and mir-148/152. On the other hand, 77 miRNAs involved in maintaining pluripotency (including mir-families: mir-371/373, mir-302/367, 200, 17/92, and C19MC) were highly represented in hPSC-EVs samples (Publication I; Tables 1 and 2). These results reflect the unique miRNA profiles of the tested cell types at their EV level.

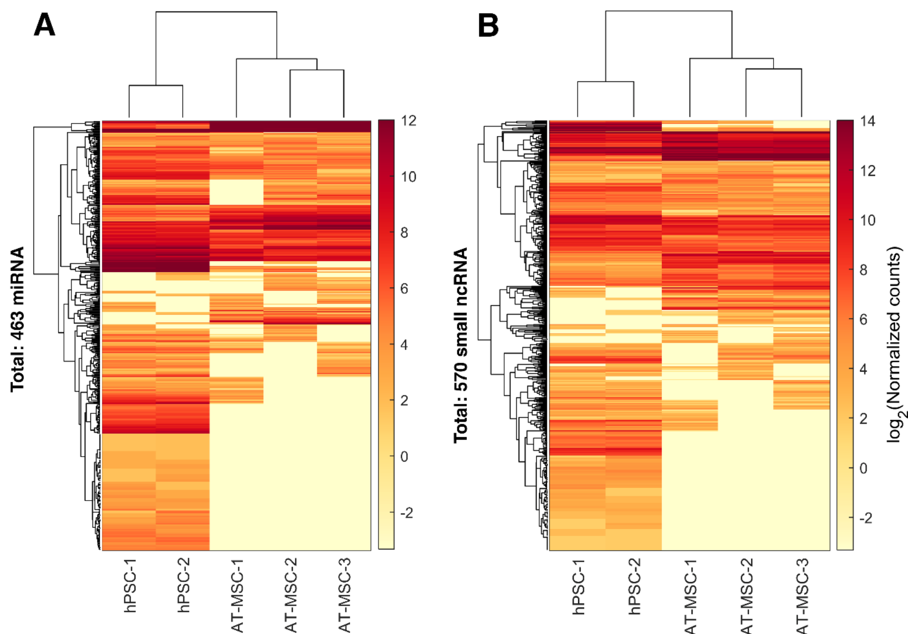

Figure 5.2: Heat map of the cluster analysis of EV-RNA. Unsupervised cluster analysis on AT-MSC and hPSC based on their EV-miRNAs (A) and ncRNAs (B). Each group clearly clustered separately and shared strong within-group similarities. Reproduced from Publication I.

5.4 Targeting HIF-1α/VEGF pathway in AT-MSCs by PHIs (Study II)

The treatment of AT-MSCs with tested PHIs (DMOG and baicalein) achieved the stabilization of HIF-1α in a comparable manner to the positive control. This stabilization response was verified by the immunostaining for HIF-1α which translocated into the nuclei of the treated AT-MSCs (Figure 5.3; A-D). The WB analysis for the cell lysates confirmed the HIF-1α stabilization and the expected VEGF expression in the treated samples as well as the positive control (Figure 5.3; E and F).

The titration of the HIF-1α stabilization response with different concentrations of DMOG and baicalein revealed a stronger response with DMOG which was, however, shorter in duration as compared to baicalein (Figure 5.3; G and H). For better reproducibility between different experimental settings, we opted to express the concentrations in (p)mole/cell for normalizing other changing parameters, e.g., plate well-size, cell number, volume of media, as was previously suggested (Doskey et al. 2015).

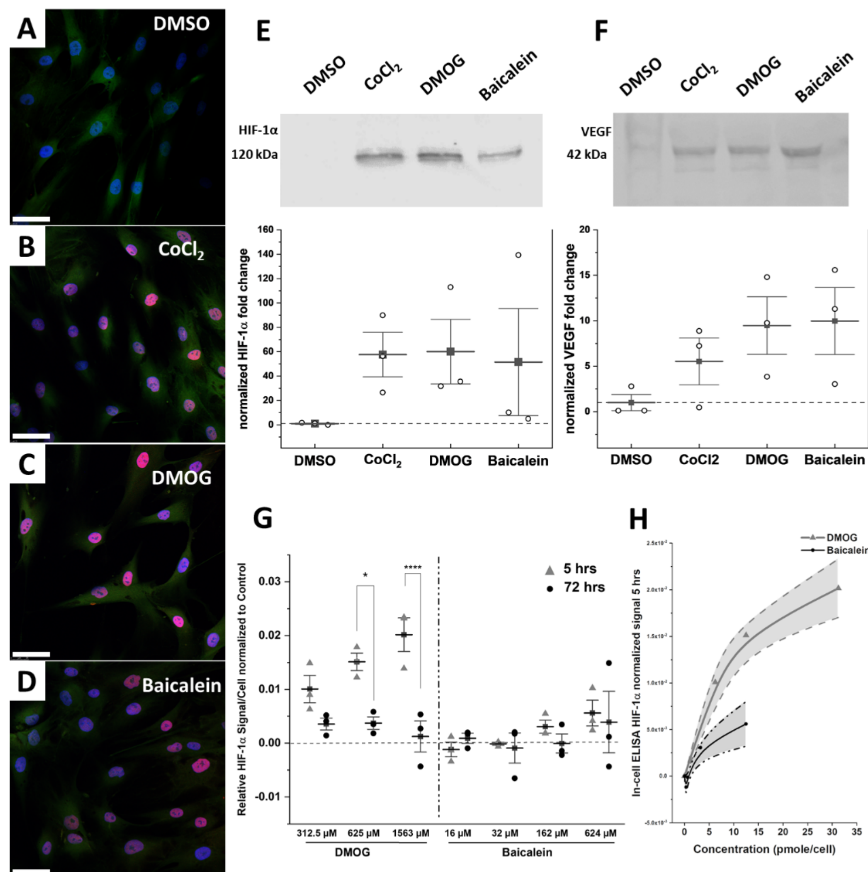

*Figure 5.3: confocal photomicrographs for HIF-1α immunostained samples of AT-MSCs treated with MM + DMSO (A) as a negative control, MM + 100 µM $CoCl_2.6H_2O$ as a positive control (B), MM + 500 µM DMOG (C), and MM + 185 µM baicalein (D). Stabilized HIF-1α translocated into the nuclei in all conditions except negative control. Western blotting of HIF-1α (E) and VEGF (F), and their band density analysis normalized to total protein stain (REVERT™) showed that both drugs as well as positive control stabilized HIF-1α and increased VEGF levels. In-Cell ELISA assay results showed a dose-dependent stabilization of HIF-1α by DMOG and baicalein at 5 hrs (H). After 72 h, HIF-1α levels declined significantly with DMOG (G). The line graph in (H) shows three donors' mean (line) ± SE (dashed areas) response for tested drug concentrations expressed in pM/cell. The box charts shows mean (▪) and SE (whiskers range) for three biological replicates (n=3), horizontal dashed reference line denotes relative signal of control condition (MM + DMSO), *$p \leq 0.05$; ****$p \leq 0.0001$. Scale bar in (A-D) = 50 µm.*

5.5 Effects of long-term PHIs treatment on AT-MSCs viability (Study II)

The viability of AT-MSCs was significantly affected by increasing concentrations of DMOG and baicalein as revealed by both CCK-8 (Figure 5.4; A) and CyQUANT (Publication II; Figure 5-D and E) assays results. Similarly, the duration of treatment had a significant impact on cellular viability (Figure 5.4; A). The highest suitable concentrations for AT-MSCs-treatment in subsequent experiments was estimated to achieve at least 70% cellular viability after 3 days of treatment. This estimation was based on the concentration/viability response curves (Figure 5.4; B). Generally, the 70% viability is considered as an accepted reference for a non-cytotoxic effect by the ISO 10993-5 standards, as was previously reported (Cannella et al. 2019, Vidal and Granjeiro 2017). The optimized concentrations were 3.75 pmol/cell of DMOG and 0.25 pmol/cell of baicalein (Figure 5.4; B).

The cell cycle analysis for AT-MSCs treated with optimized concentrations of PHIs revealed that they moderately increased the percentage of cells in G2/M phase at the expense of G0/G1 phase. The Annexin V/PI flow cytometric analysis results confirmed that those concentrations did not cause cellular apoptosis or necrosis up to 14 days of treatment compared to DMSO control (Publication II; Figure 6).

5.6 PHIs halted the osteogenic differentiation of AT-MSCs (Study II)

AT-MSCs were treated for 14 days with MM or OM, each with or without PHIs. Both DMOG and baicalein have significantly reduced the osteogenic differentiation response. In contrast to the OM+DMSO control, the PHIs treatment was associated with reduced ALP activity, relative hydroxyproline/collagen content, and mineralization of the ECM as detected by ARS staining (Figure 5.5).

5.7 Cytokine array analysis for treated AT-MSCs' conditioned media (Study II)

The cytokine array included 80 cytokines, of which 75 were detected in at least one sample and were included in our analysis. The unsupervised clustering analysis showed distinctive effects of DMOG and baicalein on cytokine levels in MM and OM (Figure 5.6; A). However, when examining the cytokines with the most robust changes in concentration levels, similar effects were observed for baicalein and DMOG, especially in angiogenesis-related cytokines. Both PHIs increased VEGF and platelet-derived growth factor-BB (PDGF-BB) levels in OM and MM media. They both increased levels of leukemia inhibitory factor (LIF) in OM and increased levels of thrombopoietin in MM (Figure 5.6; B).

However, there were also clear differences between DMOG and baicalein in the cytokine profile. DMOG decreased the concentration of several CC chemokines, such as macrophage inflammatory protein-1b (MIP-1b), macrophage-derived chemokine (MDC), eotaxin, and CXC chemokines like growth regulated α protein (GRO-α) and granulocyte chemotactic protein 2 (GCP-2). On the other hand, baicalein increased levels of stem cell factor (SCF), macrophage inflammatory protein-3 (MIP-3α), and

transforming growth factor-beta 2 (TGF-β2) in both MM and OM, and also increased the concentration of transforming growth factor-beta 3 (TGF-β3) and osteopontin in OM (Figure 5.6; B).

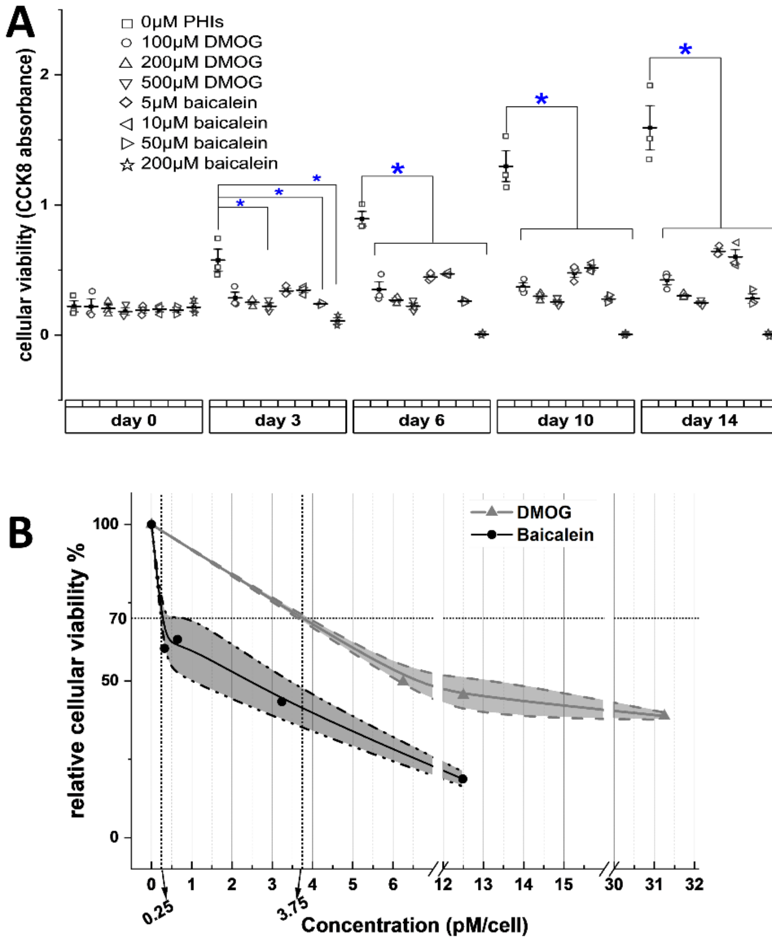

Figure 5.4: The cellular proliferation and metabolic activity deteriorated significantly with higher concentrations and longer duration of treatment with DMOG and baicalein as shown by CCK-8 assay results (A). The cellular viability response/concentration curve at the third day (B) helped to estimate the suitable concentrations for subsequent experiments. The box charts show mean (-▪-) and SE (whiskers range) for three biological replicates (n=3), horizontal dotted reference line depicts the aimed 70% viability level; corresponding drug concentrations were employed in subsequent analyses, $p \leq 0.05$ (*) compared to relative signal of control condition (MM + DMSO). The line graph in (B) shows three donors' mean (line) ± SE (dashed areas) response for tested drug concentrations expressed in pM/cell.

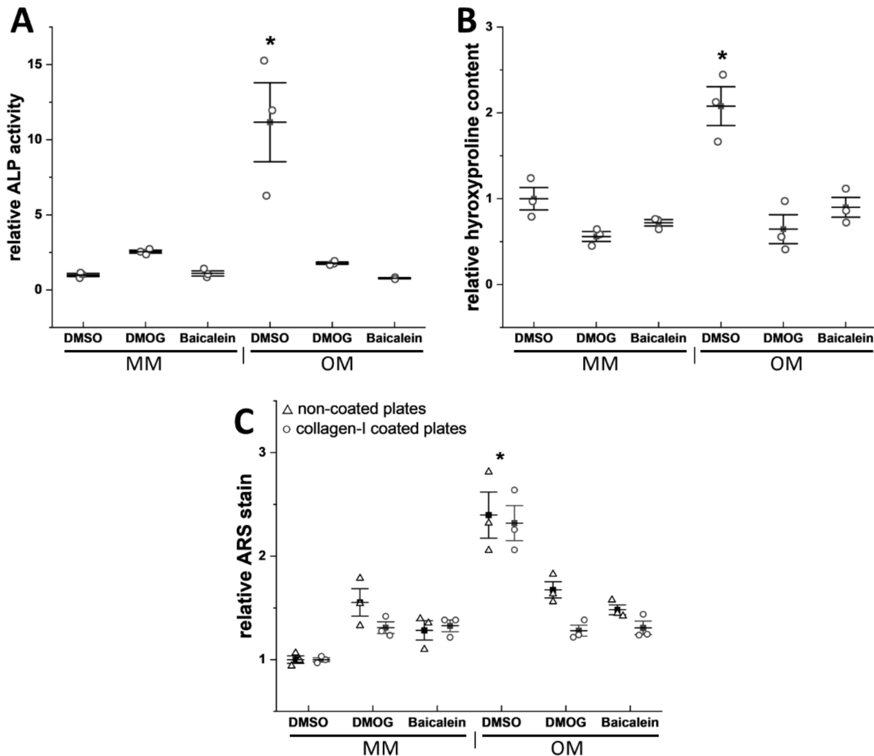

Figure 5.5: Both the tested PHIs (DMOG and baicalein) significantly reduced the osteogenic response of AT-MSCs to levels comparable to their non-osteogenic induction counterparts as revealed by alkaline phosphatase assay (ALP) (A), hydroxyproline assay (B), and alizarin red S (ARS) stain (C). The mineralized ECM was sharply reduced, as detected by ARS stain, regardless of the precoating of plates with collagen-I (C). The box charts show mean (-▪-) and SE (whiskers range) for three biological replicates (n=3), $p \leq 0.05$ (*).

5.8 Quantitative real-time PCR (qRT-PCR) results (Study II)

The treated AT-MSCs gene expression of *VEGFA* was upregulated especially with DMOG (Figure 5.7; A). The *ALPL, RUNX2,* and *COL1A1* gene expression was upregulated only by OM+DMSO control condition, while treatment with DMOG and baicalein negated such upregulation with OM (Figure 5.7; B-D). *BMP2* and *SPP1* were upregulated, however, with baicalein in both MM and OM (Figure 5.7; E and F).

The analysis of stemness-related genes expression revealed an upregulation of *KLF4* with DMOG in MM and OM, and with baicalein in OM (Figure 5.7; G). *NANOG* was upregulated with DMOG in OM and with baicalein in MM and OM (Figure 5.7; H), while *OCT4* was relatively upregulated by both PHIs in both MM and OM (Figure 5.7; I). In another setting, the upregulation of stemness-related genes; *NANOG* and *OCT4*, was consistent with repeated passaging of AT-MSCs under the continuous treatment with DMOG and baicalein in MM (Figure 5.7; J-L).

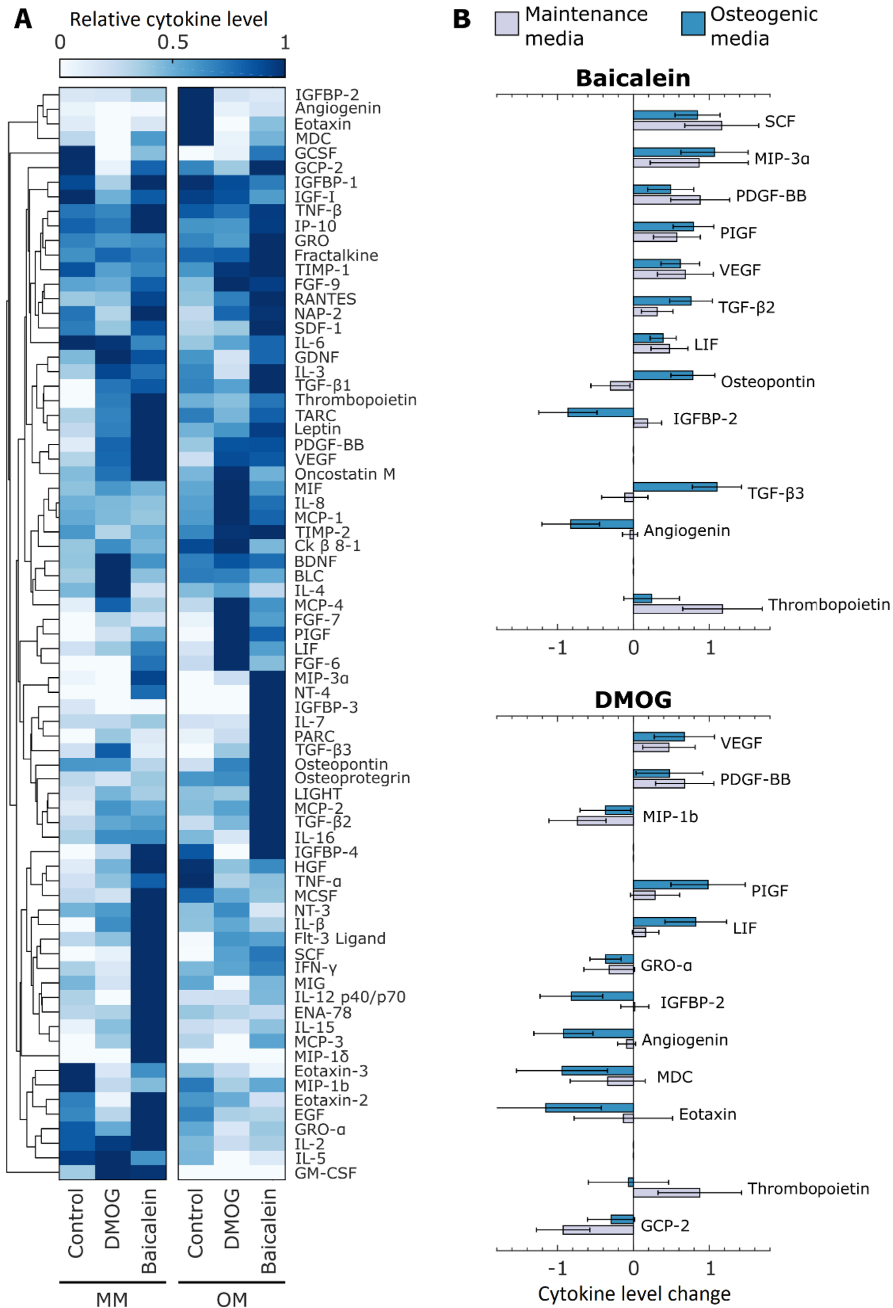

Figure 5.6: Cytokine array analysis results showing unsupervised clustering analysis of the detected cytokines (A) and relative change in concentration of 12 most significant cytokines induced by baicalein and DMOG (B). In (B), cytokines are grouped in three groups: Top = change of concentration levels in both maintenance media (MM) and osteogenic media (OM), middle = change of concentration levels only in OM, bottom = change of concentration levels only in MM. The figure is reproduced from Publication II.

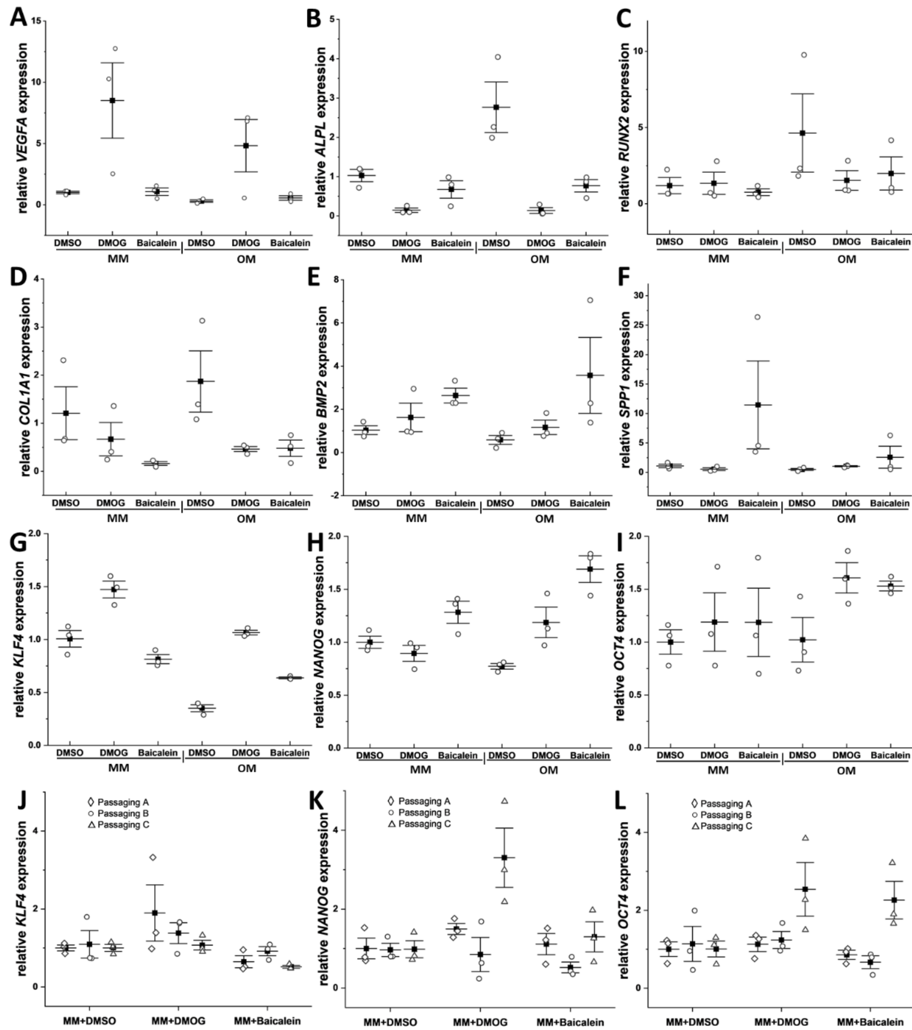

Figure 5.7: qRT-PCR results showed that DMOG and baicalein treatment for AT-MSCs upregulated their VEGFA expression (A), and downregulated osteogenesis related genes; ALPL (B), RUNX2 (C), and COL1A1 (D). Baicalein upregulated BMP2 (E) and SPP1 expression (F). PHIs treatment was associated with the upregulation of stemness-related genes; KLF4 (G), NANOG (H), or OCT4 (I), which was more evident with OM conditions. The upregulation of stemness-related genes, however, was seen under PHIs treatment in MM for successive passages (J-L) especially for NANOG (K) and OCT4 (L). Passaging A refers to first assessed passages (P4 of two donors and P5 for the third donor), the analysis of subsequent passages was carried out till passaging C (i.e., P6 for two donors and P7 for the third donor). The results showed an upregulation trend in OCT4 and NANOG with both PHIs in the latest passage. The box charts show mean (▪) and SE (whiskers range) for three biological replicates (n=3).

5.9 Assessment for TEB flap prefabrication phase (Study III)

The pre-reconstructive CTA showed clear vasculature around the implanted BBs in all groups (Figure 5.8; A and B). The quantification of the vascularization from CTA did not show significant differences among the tested IVBs. Histologically, the IHC-P, however, revealed a higher percentage of vWF-positive cells (positive%) and increased blood vessel density in MVP-group sections (Figure 5.8; C-H). The MTC-stained sections showed no signs of ectopic bone formation at the end of the prefabrication phase. During the reconstructive surgery, the prefabricated TEB flaps showed obvious vascularization of the prefabricated TEBs with bleeding through the biomaterial pores (Figure 4.3; C).

5.10 The CT and µCT analysis for the mandibular reconstruction (Study III)

The CT analysis revealed active new bone formation and biomaterial degradation between the two timepoints (Figure 5.9; A and B). The newly formed bone volume (NB/TV%) increased between the two timepoints within all groups with a corresponding decrease in the residual biomaterial volume (RM/TV%). In M-group the residual biomaterial volume was higher at terminal endpoint compared to MVP-group in both CT and µCT analysis. More new bone volume was observed in MVP-group (Figure 5.9; B and C). The reconstructed 3D models comparison (Figure 5.9; D) reflected the remodeling changes of the BBs within the tested IVBs, which was higher in MVP-group.

5.11 The histological analysis (Study III)

The histological analysis was performed on coronally-cut sections from four segments covering the defect areas (anterior, mid-anterior, mid-posterior, and posterior segments). The analysis focused on the newly formed osseous tissues, the degradation of the biomaterial, the vascularization of the fibrovascular stroma, and the changes in the muscular tissues of the prefabricated flaps.

There were some heterogenous individual responses within groups, however, the newly formed bone followed consistent patterns. Most of the bone formation arose from the bony edges of the defect and its related periosteum. The lingual periosteum showed higher capacity towards new bone formation in all groups (Figure 5.10; A-D). Nevertheless, new bone islands were frequently found within the bioimplants with no relation to the native periosteum at the defect site or the defect edges. These bone islands were more evident in both MP and MVP groups (Figure 5.10; C and D).

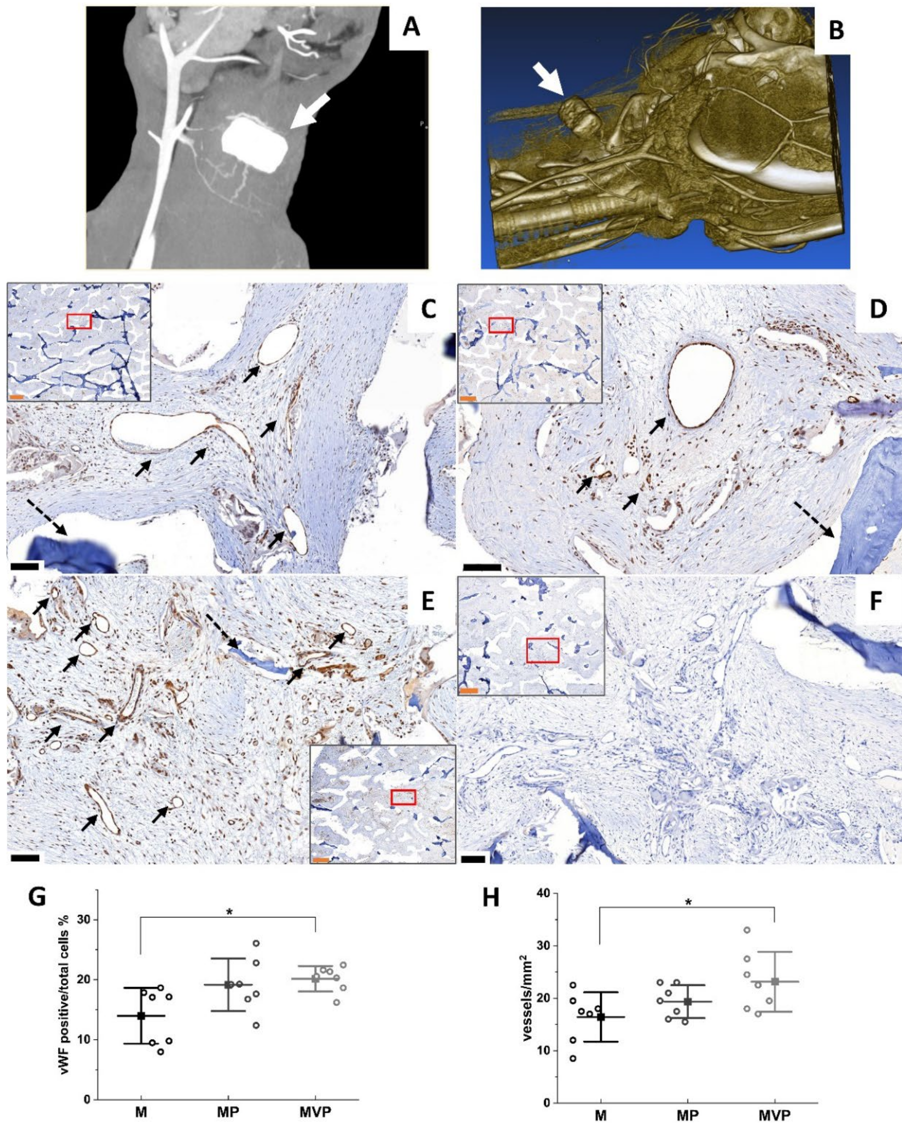

Figure 5.8: The CTA and its 3D reconstruction depict the vascularized prefabrication site in one of M-group sheep (A and B). IHC-P for vWF and density of blood vessels in the prefabricated TEB samples of different IVBs: M (C); MP (D); MVP (E); and negative IHC-P control (F) after prefabrication phase. More vascularization was seen in MVP samples especially when compared to M samples. The black arrows show the detected blood vessels, dashed arrows show residual biomaterial. Orange scale bars in section overview = 1000 µm, in higher magnification (for red boxes) the black scale bars = 100 µm. The boxplots show mean (-▪-), SD (whiskers), and averaged measurements from segments of the BB samples (o), $p<0.05$ (*). Figure is reproduced from Publication III.

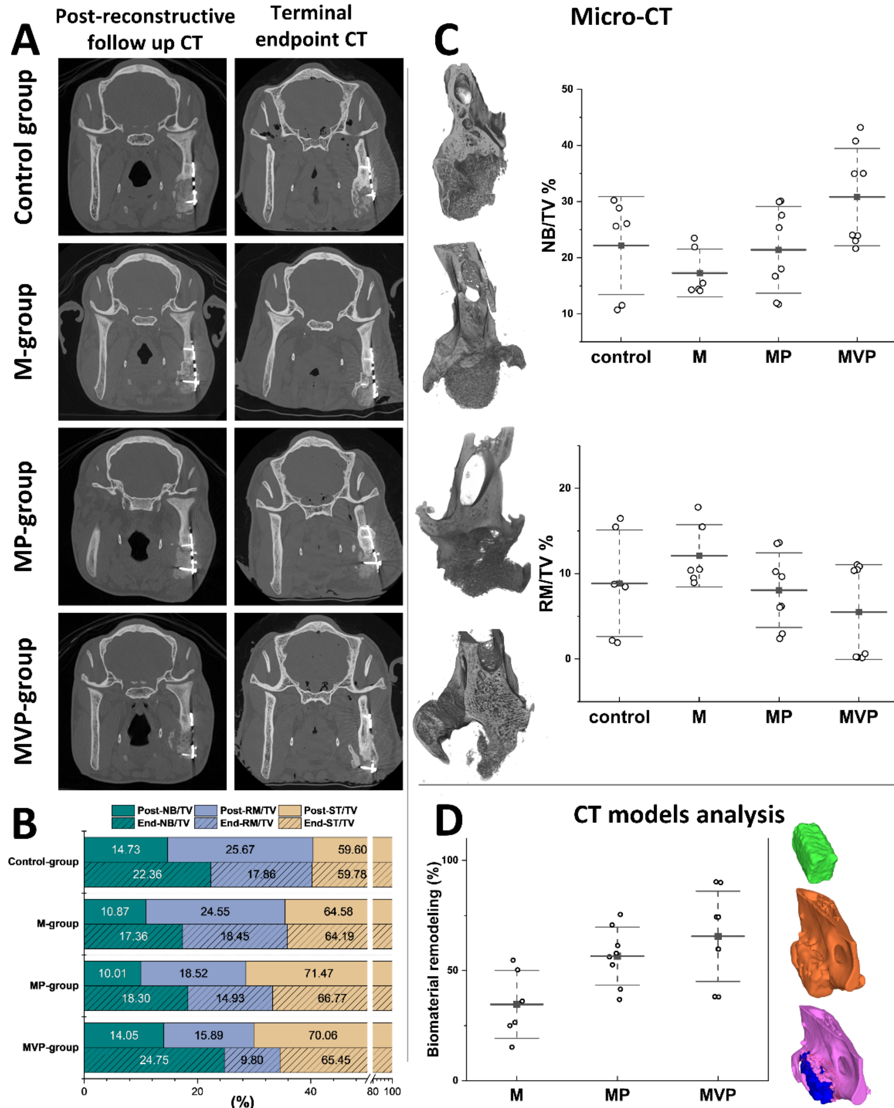

Figure 5.9: CT and µCT analyses for the reconstructive phase. New bone formation and corresponding biomaterial degradation proceeded between the two CT-timepoints (A and B). Both terminal CT and µCT showed more new bone volumes (NB/TV%) and less biomaterial volumes (RM/TV%) with MVP-group. The largest difference was seen between MVP and M groups, while MP and control groups showed comparable results (B and C). The comparison of reconstructed 3D volumes revealed higher remodeling rates in MVP-group (D). Representative 3D models (D) depict bone block from TEB flap before transplantation (green upper model), TEB reconstructed mandibular defect (gold middle model), and residual biomaterial (blue) at terminal state model (the lower model). The stacked bar chart (B) shows the average measured new bone (NB/TV%), residual biomaterial (RM/TV%), and soft tissue (ST/TV%) volumes in two timepoints CTs. The boxplots show mean (-▪-), SD (whiskers), and technical replicates (o). The $n_{Control}$=3, n_M=3, n_{MP}=4 and n_{MVP}=4. Figure is reproduced from Publication III.

The areas occupied by the newly formed bone with its marrow spaces and those occupied by the residual biomaterial and fibrovascular stroma were measured. The M-group showed the least new bone formation area (mean% ± SD) (32.05 ± 10.89), while the control-group (45.36 ± 17.81) and MVP-group (49.37 ± 14.65) showed the highest (Figure 5.10; I). Dividing the newly formed bone into bone islands and bone from the defect edges/periosteum, showed slightly more areas of bone islands in MP and MVP groups (Figure 5.10; J). The increased new bone formation in the control and MVP groups was associated with higher degradation of the biomaterial and less residual biomaterial especially in the MVP-group (Figure 5.10; J).

New ingrowing intramembranous bone infiltrated and enveloped the biomaterial (Figure 5.10; A and C). Visualized by picrosirius red and reticulin, perforating fibers with similarities to Sharpey's fibers appeared to penetrate the newly formed bone, radiating towards the degrading biomaterial and its fibrovascular stroma (Figure 5.10; D-G). The new woven bone, especially originating from the defect edges along these fibers, was more evident where biomaterial degradation was pronounced (Figure 5.10; E).

The reaction towards the biomaterial and its degradation displayed no qualitative differences. The biomaterial trabeculae had occasional multinucleated giant cells (MNGCs) on their surfaces, some of which showed osteoclast type features (Figure 5.10; E). The fibrovascular connective tissue stroma filled the biomaterial spaces and was significantly more vascularized in the MVP-group (Figure 5.10; H) whereas it was more fibrotic in M-group (Figure 5.10; B). The muscular tissues of the prefabricated flaps were mostly converted into adipose tissue with evident perivascular fatty infiltration (Figure 5.10; B and D).

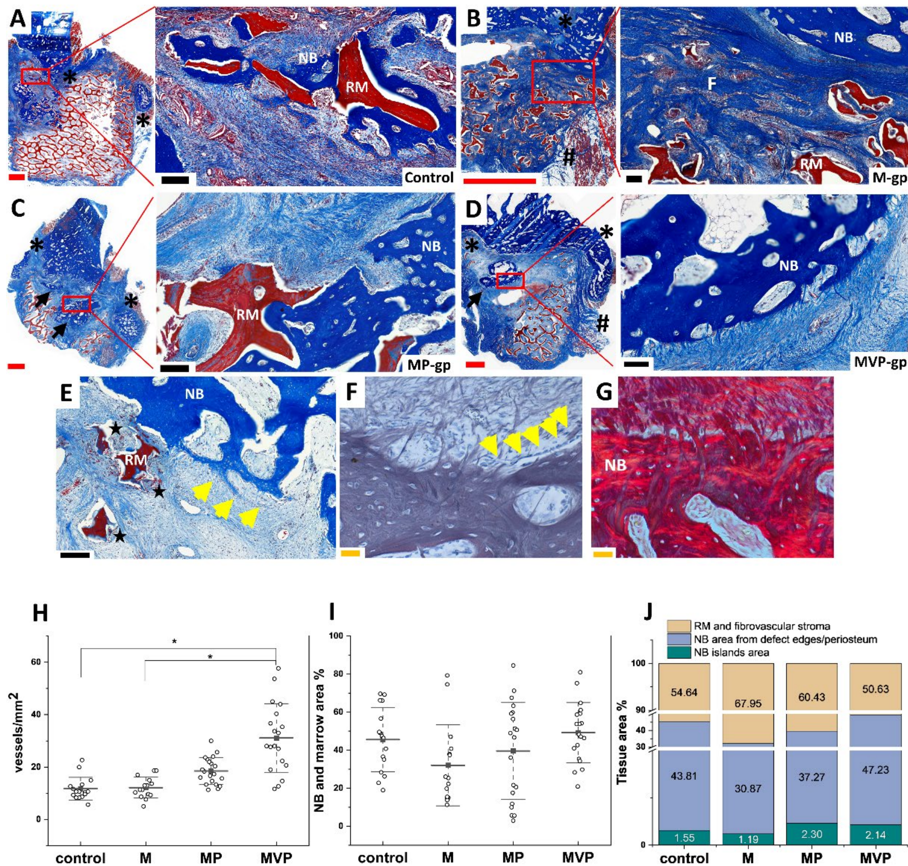

Figure 5.10: The histological findings in sheep endpoint samples. Representative sections were stained by MTC (A-E). New bone extended from the defect edges/periosteum (✱), especially lingually. Bone islands (black arrows) were frequently seen in MP and MVP groups. The ingrowing intramembranous new bone (NB) infiltrated and enveloped the biomaterial (RM). Fibrotic stroma (F) was evident in M-group (B). The perivascular fatty infiltration (#) was seen in the muscular components of the prefabricated flaps (B and D). Speckled perforating collagen fibers were seen related to the newly formed bone (D-G). These Sharpey's-like fibers were evident in decalcified sections stained with reticulin (F) and picrosirius red under polarized light (G). The newly formed bone followed related active osteoblasts (yellow arrow heads). The biomaterial degradation foci (E) show groups of macrophages and MNGCs (★) with remnants of biomaterial. Boxplots show mean (-▪-), SD (whiskers), and technical replicates (o). The stacked column chart (J) shows the average measured areas in histological sections. The red scale bars in WSIs = 2000 µm, black scale bars in higher magnification (of red boxes) = 200 µm, yellow scale bars (F & G) = 50 µm. The $n_{Control}$=3, n_M=3, n_{MP}=4 and n_{MVP}=4, $p \leq 0.05$ (*). Figure is reproduced from Publication III.

6 DISCUSSION

Craniomaxillofacial bone defects represent an enduring clinical challenge. Such defects frequently exceed the inherent regenerative capacity of bone causing a significant deformity and functional disability (Spanjer et al. 2017). Bone tissue engineering (BTE) has received a considerable attention derived by the need for expanding the reconstructive armamentarium. The clinical translation of BTE, however, has not advanced without hurdles, which fed a view for BTE being over-promised and under-delivered from a clinical perspective (Mastrullo et al. 2020, Nerem 2006). The current translational approaches advance towards cell-free techniques, more stable and controllable bioactive agents (e.g., small molecules), and overcoming the BTE vascularization limitations.

EVs represent an emerging approach for cell-free BTE techniques. MSCs-EVs have been proven to have comparable potency and effects to the MSCs (Qiu et al. 2018). They play an active role in intercellular communication and in the controlling of cellular functions (Kaur et al. 2019, Marolt Presen et al. 2019). Current EV research should therefore focus on determining the best cell source for EV production and the potential modes of action (Marolt Presen et al. 2019). The bioactive cargo of EVs is complex and depends on their cell of origin. This cargo includes proteins, mRNA, miRNAs, DNAs, and lipids (Kaur et al. 2019). There is a growing evidence that the bioactive effects of EVs on target cells depends mainly on their intravesicular miRNA for gene regulation (Cantaluppi et al. 2012, Diehl et al. 2012, Pfeifer et al. 2015, Qiu et al. 2018).

In this thesis, study I involved the characterization of the EV-derived miRNAs and other small ncRNAs from two cell sources, which are AT-MSCs and hPSCs. The scarcity of in-depth characterization studies for EV-derived ncRNAs drove our interest in these clinically relevant cells. The results showed distinctive small ncRNA profiles of AT-MSCs and hPSCs, which highlight the effect of the donor cells on the EV-derived miRNAs, as previously reported (Billing et al. 2016). The miRNAs in hPSCs-EVs included some characteristic human PSCs miRNAs, e.g., miR-302, miR-372, mir-17-92, mir-200, and C19MC families (Wilson et al. 2009). These were found in both hESCs and hiPSCs EVs, which suggest that hPSCs, irrespective of their derivation, release their characteristic miRNAs in the EV cargo.

Clark and coworkers have presented a global miRNA expression profile of MSCs from different sources (Clark et al. 2014). Among these consensus miRNAs, mir-199a, 152, 125a, b, 143, 100 and let-7b, c, e, f, were seen in our AT-MSCs derived EVs. Additionally, mir-10a-5p, mir-10b-5p, mir-22-3p, mir-143-3p, mir-100-5p, let-7a, f-5p identified in our AT-MSCs EVs conformed to previous reports, indicating that these miRNAs are consistent among different donors (Baglio et al. 2015). Moreover, let-7a-5p, let-7b-5p, let-7i-5p, miR-125-5p, miR-199a-3p, miR-199b-3p, miR-100-5p, miR-144-3p and miR-22-3p among the highly expressed in AT-MSCs EVs have been previously found in BMSCs EVs (Ferguson et al. 2018). These findings indicate that

certain signature EV miRNAs are not affected by the donor variations or the cell of origin, thus highlighting the regulatory potential of EV-ncRNAs and the potential stem cell specific EV biomarkers for future studies and applications.

Previous reports have demonstrated the role of miRNAs in the regulation of osteogenesis and bone remodeling. This role involves enhancing osteoblastic differentiation by targeting osteogenesis negative regulators and vice versa (Kapinas and Delany 2011, Papaioannou et al. 2014). In our data, only AT-MSCs EVs showed EV-miRNAs which are related to osteogenic differentiation. EV-miRNAs which modulate osteogenic commitment and promote osteogenesis of MSCs included let-7a and c, mir-22, 199a, 196a, 199b (Shan Huang et al. 2012, Kim et al. 2009, Wei et al. 2014, Zhao et al. 2016). Negative regulators of osteogenic differentiation were also seen, such as mir-27, 98, 100, 615, 125b and 195 (Almeida et al. 2016, Hassan et al. 2010, Wang et al. 2017, Yin et al. 2017, Zeng et al. 2012). Thus EV-miRNAs which are negative regulators of osteogenesis could be the potential targets for enhancing bone regeneration by delivering anti-miR oligonucleotides into bone injury sites. It is known that EVs protect miRNAs in fluids from circulating RNAses (Boon and Vickers 2013, Pfeifer et al. 2015). Future approaches should explore the use of EVs as vehicles to deliver miRNA mimics or anti-miRs. This could be a promising tool in future bone regeneration applications.

After the proof of EV-mediated MSCs signaling, last decade has witnessed an intensive research to explore the EVs potential in the development of biologics for tissue repair (Lai et al. 2010). EVs are more promising than cell-based approaches due to the fact that they are not self-replicating, are less responsive to the local environments, and easily handled and sterilized by filtration. However, the clinical translation for EV-based approaches requires the optimization and standardization for *in vitro* culture of source cells, harvesting, purification methods, characterization, quality control, dosage, and potency assays. It has been shown that the *in vitro* culturing conditions and especially the serum supplements can affect the EV yield and interfere with the EVs produced by the cells (Kornilov et al. 2018). Additionally, all EV isolation methods result in a mixed population of vesicles with varying yield and potential co-isolation of soluble protein by less-stringent purification protocols (Whittaker et al. 2020). One key approach to tackle these concerns is to develop reproducible potency assays for EVs. The optimization of upstream and downstream bioprocessing should be performed with respect to function (Whittaker et al. 2020). This will facilitate the regulatory aspects for clinical translation of EV-based approaches.

Most advances towards clinical applications of tissue engineering have been relatively restricted to thin or avascular tissues, e.g., skin, cartilage, and cornea. Vascularization remains a critical challenge for large tissue engineered constructs (Mastrullo et al. 2020, Novosel et al. 2011). It is, therefore, reasonable to employ angiogenic factors, e.g., VEGF, for inducing neovascularization in engineered tissues.

However, as these factors suffer from a high *in vivo* instability, research efforts are ongoing to replace bolus delivery by area-restricted and long-term delivery strategies (Novosel et al. 2011, Santos and Reis 2010, Simón-Yarza et al. 2012). These strategies aim to overcome the high degradation rate of the expensive growth factors by integrating them in new biomaterial strategies which feature spatially and temporally controlled delivery (Novosel et al. 2011). Obviously, this raises the already high expectations for suitable biomaterial properties and complicates their production process. Hence, small molecules stand out as interesting alternatives due to their stability, low cost, independence of complex molecular 3D structures, and non-immunogenic characteristics (Schliephake and Böhrnsen 2019). These molecules may prove to be easier to integrate with a wide range of biomaterials.

Study II of this thesis focused on exploring the HIF-1α/VEGF pathway in AT-MSCs by testing the PHI activity of two commercially available small molecules; DMOG and baicalein. Both DMOG and baicalein have shown evident PHI effects on AT-MSCs *in vitro*, as manifested by HIF-1α stabilization under normoxic conditions and the corresponding upregulation of VEGF. However, they also showed antiproliferative effects on AT-MSCs in a dose- and time-dependent manner. The effects of hypoxia-inducible factors on cellular proliferation are cell type- and context-dependent (Hubbi and Semenza 2015). Hypoxia has diverse impacts on various physiological processes, which include angiogenesis, erythropoiesis, metabolism, and autophagy. It has been suggested that hypoxia decreases cellular proliferation as an adaptive mechanism to alleviate hypoxic stress by decreasing the oxygen demand. However, some cell populations can maintain cellular proliferation under hypoxia, which is a hallmark of cancer pathology, as well as the physiologic maintenance of stem cells in their niches (Hubbi and Semenza 2015).

In our setting, PHIs concentrations had a critical effect on cellular response. As a reference, we used sub-toxic doses of DMOG and baicalein, which maintained at least 70% viable cells as compared to the control DMSO. These concentrations allowed studying the influence of long-term treatment with tested drugs in OM or MM up to 14 days. After 14 days, no significant apoptosis was seen, the cell cycle analysis revealed a moderately increased percentage of cells in G2/M phase at the expense of G0/G1 phase. This delay or slow-down in the cell cycle is expected to be invested in proofreading DNA duplication properly prior to mitosis as suggested previously (Khamchun and Thongboonkerd 2018).

The HIF-1α/VEGF pathway is proposed to play a critical role for angiogenesis-osteogenesis coupling during developmental and postnatal bone regeneration (Grosso et al. 2017). The mechanism of this role seems to be complex and context-dependent. Targeting the HIF-1α pathway in osteoblastic lineage cells showed variable responses. Rat BMSCs were shown to undergo osteogenic differentiation in response to hypoxia (Lennon et al. 2001). Wagegg and coworkers reported enhanced osteogenesis of human BMSCs by physical and chemically-induced hypoxia when

PHIs were applied once weekly (Wagegg et al. 2012). On the other hand, other studies have demonstrated hypoxia to exhibit no or even negative effects on osteogenesis by BMSCs and osteoblasts (D'Ippolito et al. 2006, Yong‐Can Huang et al. 2012, Potier et al. 2007, Salim et al. 2004, Zhang et al. 2017).

Making comparisons among these previous studies is complicated due to the heterogeneity of the tested cell sources, species, media, and duration of treatment as previously reported (Lennon et al. 2001). Moreover, most studies report the effects of hypoxia which is achieved by three-gas modular incubators, with which fluctuations in oxygen concentration could be expected at least during medium changes and regular checking under phase-contrast microscope. These possible intermittent hypoxic conditions add to the complexity of interpreting and reproducing previous results. The heterogeneity of experimental settings is also seen in experiments using hypoxia-mimicking agents which are sensitive to concentration changes (Wu et al. 2013, Zhang et al. 2016). In our setting, we emphasized the importance of integrating different physical parameters of the experiment in effective drug concentration calculation, e.g., cell-seeding density, plate well-size, and volume of the media. Expressing the applied concentrations in mole/cell can improve translatability of experiments between labs as suggested previously (Doskey et al. 2015).

DMOG and baicalein significantly reduced the osteogenic differentiation response of AT-MSCs. We further investigated the effects of cobalt chloride ($CoCl_2$) in a similar experimental setting. $CoCl_2$ is a standard chemical inducer of hypoxic response in cell culture (Qing Li et al. 2018, Wu and Yotnda 2011). Our findings showed, that $CoCl_2$ paralleled DMOG and baicalein in the tested concentrations for slowing AT-MSCs proliferation and attenuating their osteogenic differentiation. This suggests a global effect related to the chemically-induced hypoxia and HIF-1α stabilization.

Almost a century ago, Otto Warburg demonstrated that tumor cells, unlike normal cells, modify their metabolic pathways to favor aerobic glycolysis and decrease mitochondrial oxidative phosphorylation (OXPHOS), irrespective of cellular oxygenation levels. This phenomenon is known as the Warburg effect (Bertout et al. 2008, Warburg et al. 1927). HIF-1 plays a critical role as a regulator of mitochondrial metabolism, and accumulating evidence suggests HIF-1 to be behind the Warburg effect by switching from mitochondrial OXPHOS to aerobic glycolysis, and by HIF-1-mediated inhibition of mitochondrial biogenesis (Nagao et al. 2019, Semenza 2011). Mitochondrial OXPHOS is important for osteoblastic differentiation. Shares and coworkers have shown that active mitochondria are mandatory to support osteoblastic differentiation by promoting β-catenin acetylation and activity. BMSCs osteogenic potential is reduced with an OXPHOS inhibitor (Shares et al. 2018). Additionally, DMOG and baicalein have been reported to act as OXPHOS inhibitors by interfering with mitochondrial function independent on activation of HIF signaling pathway (Huang et al. 2020, Zhdanov et al. 2015). Therefore, the observed

attenuation of osteogenic potential with the tested PHIs could be due to HIF-dependent and/or HIF-independent metabolic changes and mitochondrial activity (Huang et al. 2020, Okamoto et al. 2017, Shares et al. 2018, Zhdanov et al. 2015). Moreover, HIF-1α has been suggested to inhibit the Wnt/β-catenin pathway, this inhibition has been shown to be, at least partially, due to the HIF-1α-mediated activation of the Wnt antagonist sclerostin (SOST) (Chen et al. 2013). In our setting, a similar trend for *SOST* upregulation, especially with DMOG treatment was evident.

Both DMOG and baicalein caused an upregulation of stemness markers *KLF4*, *NANOG*, *and OCT4* in AT-MSCs. In another setting, similar findings were observed when treating AT-MSCs with PHIs in MM for successive passages. This upregulation was evident in later passages especially for *NANOG* and *OCT4*. The mechanism behind such upregulation needs further investigation, however, an interplay of the aforementioned mechanisms especially involving the modulation of mitochondrial activity and metabolism could be implicated as previously has been suggested (Papa et al. 2019).

The overall findings in study II show that prolonged chemically-induced hypoxic response in AT-MSCs inhibits their osteogenic potential and rather directs the cells towards favoring stemness phenotype. This reflects an intimate correlation between stemness and prolonged hypoxic response, which mimics the hypoxic niches of MSCs, where they maintain their stemness and self-renewal properties. Treating AT-MSCs with hypoxia-mimicking agents, e.g., DMOG and baicalein, enhanced their proangiogenic properties as evidenced by their cytokine release of proangiogenic factors, e.g., VEGF and PDGF-BB. Meanwhile, treated cells expressed other factors which are critical for homing of MSCs, e.g., SCF and TGF-β2 (De Becker and Riet 2016). These paracrine factors together with the metabolic, proliferative, and stemness modulation could improve AT-MSCs survival and engraftment *in vivo* and boost their regenerative potential.

The predictable application of *in vivo* tissue engineering techniques can achieve a paradigm shift in bone reconstructive approaches. Some reconstructive approaches have been reported to employ the patient own body for staged cutaneous flap prefabrication, total nasal prefabrication, and calvarial bone intracorporeal preservation (Jeyaraj 2020, Lazzeri et al. 2013, Robiony et al. 2011). This approach is promising for the clinical translation of BTE through the IVB strategy which should be based on an understanding of the regenerative potential and limitations of different tissues/elements employed in IVB strategies. In a preclinical ovine model, study III investigated the performance of muscular and periosteal IVBs for prefabricating vascularized myoosseous flaps when combined with a cell-free alloplastic biomaterial. This approach appreciates the importance of minimizing the use of *in vitro* expanded cells and exogenous growth factors for an ideal IVB strategy (Huang, Kobayashi, et al. 2016).

The findings of study III revealed that the prevascularization of alloplastic bone blocks was successful in the brachiocephalic muscle pouches with or without periosteal grafts/flaps. However, the employment of vascularized periosteal flaps did achieve more robust vascularization as compared to other IVB techniques. After a flap prefabrication period of 9 weeks, no evidence of ectopic osteogenesis was seen in any of the tested IVB techniques. However, both the periosteal grafts and periosteal flaps enhanced the performance of the prefabricated TEB flaps in the reconstruction of mandibular CSDs after transplantation.

The osteogenic potential of periosteum was reported already three centuries ago (Hutmacher and Sittinger 2003). Today, this unique tissue is still inspiring reconstructive surgeons to induce the formation of a similar membrane which can provide vascularization, growth factors, and MSCs to bone defects via the increasingly popular Masquelet induced membrane technique (Masquelet 2017, Masquelet and Begue 2010, Piacentini et al. 2019). In the tested ovine model, the pericranium was used since it is a clinically relevant source for harvesting larger periosteal tissues (Battaglia et al. 2020). The dependence of the periosteal osteogenic capacity on a viable osseous tissue environment has been debated in the literature. In a dog model study series, Burstein and Canalis suggested that the osteogenic capacity of the periosteum depends on both the maintenance of its blood supply and the interaction with viable bone (Canalis and Burstein 1985). They have, however, later reported that periosteal tissues, when transferred into soft tissues away from a significant bone-periosteal contact, were able to induce osteogenesis. They concluded that an intimate bony-periosteal contact is not a precondition for periosteal osteogenesis but rather can boost bone formation rate (Burstein and Canalis 1985). In our study, periosteal grafts and flaps did not induce ectopic osteogenesis of biomaterials in the employed muscle pouches despite achieving a robust vascularization. However, when transplanted into the mechanically stimulated mandibular defects, both the periosteal graft and periosteal flap wrapped BBs induced more bone islands as compared to other groups. This reflects the importance of the bony microenvironment and mechanical stimulation for the osteogenic potential of periosteal tissues.

Ersoy and coworkers have studied the ectopic osteogenic potential of periosteal tissues in a murine model (Ersoy et al. 2015). The saphenous artery periosteal island flaps were transposed to abdomen and they were insufficient for osteogenesis when applied solely without additional osteoinductive agents (Ersoy et al. 2015). A similar observation was found in our ovine model, however, the periosteal tissues seemed to preserve some osteogenic potential with adequate blood supply in soft tissues. Such osteogenic potential is manifested under mechanical stimulation in a bony microenvironment. Huang and coworkers reported enhanced bone formation on demineralized bone matrix scaffolds when applied in pedicled pericranial flaps in a rabbit model (Huang et al. 2017). To be noted, however, that the employed pericranial flap in this rabbit model study was closely related to the native skull bone.

This could support the suggested impact of the local bony environment on the osteogenic capacity of periosteal tissues. On the other hand, Huang and coworkers reported osteogenesis occurring in scaffolds in muscular pouches of rabbits at 8 and 16 weeks (Huang et al. 2017). In our model, no osteogenesis occurred in sheep muscular pouches after 8-11 weeks, which could reflect a different osteogenic response in different species or could be due to the different muscles employed, also the activity of the used biomaterial could play a role. The periosteal tissues were previously suggested to act as bioactive membranes, while providing a simultaneous guided bone regeneration effects (Dimitriou et al. 2012, Huang, Kobayashi, et al. 2016). The findings of study III in this thesis support this role, as the muscular pouches IVBs (M-group) were associated with less vascularization, decreased new bone formation, slower biomaterial remodeling, and relatively more stromal fibrosis.

Biomaterial degradation/resorption is crucial for allowing the ingrowth of new bone and vascular tissues. Large amounts of residual biomaterial, regardless of its biocompatibility, were shown to be associated with later complications, e.g., infection, and poor outcomes (Heliotis et al. 2006, Z. Sheikh et al. 2015). The vascularization was suggested to be one of the controlling factors for biomaterial degradation. Wu and coworkers reported higher degradation rate in β-TCP scaffolds with enhanced vascularization by an arteriovenous loop as compared to a vascular bundle in beagle dogs (Wu et al. 2017). According to the results in study III, more biomaterial degradation was seen in MVP-group showing robust vascularization, however, a comparable degradation was found also in the least-vascularized control-group. Considering the shorter *in vivo* time for biomaterials in the control-group, it is suggested that the mechanical stresses in the recipient site played a principal role in biomaterial resorption together with vascularization.

The role of periosteum-Sharpey's fibers-endosteum system has been previously suggested for trabecular generation in both developmental and regenerative bone (Aaron and Skerry 1994). In a sheep model, Aaron and Skerry showed that the injured endosteum/periosteum extends arrays of distinct collagenous fibers, which migrate into the healing site to form a preliminary polarized framework supporting the developing bony trabeculae. In our sheep model, these perforating Sharpey's fibers were evident elements in relation to the newly formed bone. They bridged the excised bony surfaces, the periosteum/endosteum, and the biomaterials, and seemed to exhibit a scaffolding effect for regenerating trabecular intramembranous ossification in accordance with previous reports (Aaron 2012, Aaron and Skerry 1994). Study III findings suggest that vascularized periosteal flaps are expected to induce more vascularization in biomaterials and higher remodeling rates upon transplantation for reconstruction. Further studies should consider the appropriate design of periosteum-based IVB strategies to allow interaction with a close bony microenvironment if osteoinductive agents are not employed. The predictability of such strategies should be warranted for expanding our reconstructive armamentarium.

7 CONCLUSIONS

The overall aim of the thesis work was to explore clinically translatable tissue engineering approaches for the management of craniomaxillofacial bone defects. We envisaged this potential in the EV-based cell-free techniques, where we identified EV-miRNA and other small ncRNA signatures released by AT-MSCs and hPSCs. This adds to our understanding of the mechanisms behind EV-mediated intercellular communication. We also addressed the vascularization challenges by investigating the potential effects of hypoxia-mimicking agents on AT-MSCs *in vitro*. Additionally, we developed a preclinical model involving the IVB strategy for the prefabrication of TEB flaps and the subsequent transplantation for reconstruction of mandibular bone defects in sheep.

Based on the presented findings, the following conclusions can be drawn:

- both AT-MSCs and hPSCs secrete a selective pattern of small ncRNA in their cell-free secretome. This could be a mechanism for maintaining the stem cell specific characteristics, orchestrating gene expression, and mediating communication between neighboring cells. The in-depth understanding of EV-derived ncRNA regulatory mechanisms could provide strategies for developing engineered EVs with therapeutic RNA. **(Study I)**

- Hypoxia-mimicking small molecules; DMOG and baicalein, are efficient for enhancing the proangiogenic potential of AT-MSCs via HIF-1α/VEGF pathway activation. This was associated with slowed proliferation, reduced osteogenic differentiation, upregulated stemness markers, and expression of MSCs homing molecules, e.g., SCF and TGF-β2 in the AT-MSCs. Taken together, these effects could enhance the survival and engraftment of AT-MSCs *in vivo* while inducing vascularization from surrounding tissues. **(Study II)**

- The tested IVB strategies achieved predictable vascularization in cell-free alloplastic bone blocks. The periosteal grafts/flaps improved the outcomes of prefabricated TEB flaps over muscle-IVB. The periosteum showed a predictable pro-vascularization and pro-osteogenic potential. The vascularized periosteal flaps had greater pro-vascularization effects compared to transplanted non-vascularized grafts. Biomaterial remodeling was enhanced in association with vascularized periosteal flaps. The osteogenic potential of periosteum, however, was not critically affected by the maintenance of its own vascular supply, but rather depended on its interaction with a mechanically stimulated local bony microenvironment after transplantation into mandibular defects. Incorporation of periosteal flaps was associated with more new bone formation, robust vascularization, and higher remodeling rates of TEB flaps upon transplantation. **(Study III)**

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