Stemness and osteogenic differentiation of induced pluripotent stem cells generated in xeno-free conditions

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"I'm reflective only in the sense that I learn to move forward. I reflect with a purpose"

- **Kobe Bryant**

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Scientific environment

The work comprising this thesis was conducted as part of the Tisse Engineering Research Group, Department of Clinical Dentistry, Faculty of Medicine, University of Bergen (UiB).

Cell isolation from collected biopsies and subsequent cell propagation was performed at Gade Laboratory for Pathology, Department of Clinical Medicine, UiB, and at the Department of Clinical Dentistry, UiB. The reprogramming of fibroblasts and subsequent propagation of generated induced pluripotent stem cells (iPS) was conducted at Glasblokkene, Haukeland University Hospital. The characterization of the iPS was performed at Glasblokkene and the Department of Clinical Dentistry, UiB. The differentiation of the iPS into induced mesenchymal stem cells (iMSC), iMSC culture, and iMSC characterization was performed at the Department of Clinical Dentistry, UiB. Animal experiments were performed at the Animal Facility, Haukeland University Hospital.

The main supervisor of the project was Professor Kamal Mustafa. The co-supervisors were Professor Helge Ræder, Professor Daniela Elena Costea and Dr. Salwa Suliman.

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Abstract in English

Mesenchymal stem cells (MSC) have long been a cornerstone in bone tissue engineering strategies, catalyzing the formation of new bone. Nevertheless, these cells generally present with challenges making adequate investigation quite difficult. MSC-like cells (iMSC) generated from induced pluripotent stem cells (iPS) have emerged as viable alternatives to traditional MSC. As the literature has shown, iMSC can be generated from different types of iPS, *i.e.*, iPS generated from different sources. However, since cell reprogramming is generally a tedious and inefficient process, it is vital to determine sources which are more susceptible to this procedure. Furthermore, for the purpose of translation, iMSC should be generated using xeno-free methods that are compliant with GMP guidelines. These include the isolation and expansion of the initial cell source, the generation and culture of the iPS, and the differentiation of iPS to iMSC. Hence, the focus of this thesis was to ultimately develop a xeno-free protocol for generating iMSC and to subsequently assess their osteogenic capacity.

In the first study, the objective was to assess and compare the osteogenic potential of BM-MSC propagated in two separate xeno-free protocols (basal media supplemented with either platelet lysate (PL) or human AB serum). Both xeno-free protocols supported BM-MSC expansion *in vitro* while also supporting their osteogenic differentiation in both *in vitro* and *in vivo* settings. Eventually PL was selected as the xeno-free supplement of choice for the remaining studies due to a variety of factors including, but not limited to, practicality, availability, and future applicability.

The second study aimed to generate iPS from different donor-matched fibroblast sources (dermal, buccal, and gingival) using a xeno-free, PL-based protocol. The generated xeno-free iPS were then compared to xenogenic iPS (FBS protocol) obtained from the same donor-matched fibroblasts. Both protocols supported the generation of iPS from the various fibroblasts, however, the fibroblasts propagated in PL showed generally lower reprogramming efficiencies. Furthermore, the oral fibroblasts generally proved to be more difficult to reprogram than the dermal fibroblasts. Upon

successful generation, the iPS geno/phenotype remained largely unaffected by both the expansion conditions and initial cell source.

The aim of the third study was to differentiate the generated xenogenic and xeno-free iPS into iMSC, and to assess and compare their osteogenic potential. Both protocols supported the differentiation of iPS into iMSC. Furthermore, the protocols supported the osteogenic differentiation of iMSC in both *in vitro* and *in vivo* settings. Finally, the initial cell source had little bearing on the eventual iMSC geno/phenotype and osteogenic potential.

In the course of this thesis, we shed light on the advantages and hurdles encountered when deriving iPS/iMSC from various origins in xeno-free conditions. Collectively, these findings warrant further evaluation and investigation.

Abstract in Norwegian

Mesenkymale stamceller (MSC) har lenge vært grunnleggende i strategiene for bein tissue engineering, og er viktige i dannelsen av nytt bein. Samtidig har disse cellene flere utfordringer som gjør grundig undersøkelse ganske vanskelig. MSC-lignende celler (iMSC) frembrakt fra induserte pluripotente stamceller (iPS) har dukket opp som levedyktige alternativer til tradisjonelle MSC. Som litteraturen viser, kan iMSC dannes fra ulike typer iPS, det vil si iPS generert fra forskjellige kilder. Men; siden celleomprogrammering generelt er en tidkrevende og ineffektiv prosess, er det viktig å finne kilder av celler som er mer mottakelige for denne omdannelsen. Videre, med tanke på translasjon, bør iMSC dannes ved hjelp av xeno-frie metoder som er i samsvar med GMP-retningslinjer. Dette inkluderer isolasjon og ekspansjon av den opprinnelige cellekilden, generering og dyrking av iPS, og differensiering av iPS til iMSC. Fokuset i denne avhandlingen var derfor å utvikle en xeno-fri protokoll for å generere iMSC og deretter å vurdere iMSC sin osteogene kapasitet.

I den første studien var målet å vurdere og sammenligne den osteogene potensialet til BM-MSC som ble dyrket opp i to separate xeno-frie protokoller (grunnleggende medier supplert med enten platelet lysat (PL) eller humant AB-serum). Begge xeno-frie protokollene støttet BM-MSC ekspansjon in vitro og støttet også deres osteogene differensiering både in vitro og in vivo. Til slutt ble PL valgt som det xeno-frie tillegget for de gjenværende studiene på grunn av forskjellige faktorer, inkludert, men ikke begrenset til, praktisk anvendelighet, tilgjengelighet og fremtidig anvendelighet.

Den andre studien hadde som mål å lage iPS fra forskjellige donor-like fibroblastkilder (dermale, buccale og gingivale) ved hjelp av en xeno-fri, PL-basert protokoll. De genererete xeno-frie iPS ble siden sammenlignet med xenogene iPS (FBS-protokoll) fra de samme donor-matchede fibroblastene. Begge protokollene støttet generering av iPS fra de ulike fibroblastene, men fibroblastene dyrket i PL viste generelt lavere omprogrammeringseffektivitet. Videre viste munn-fibroblastene seg generelt å være vanskeligere å omprogrammere enn dermale fibroblaster. Ved vellykket generering

forble iPS geno/fenotype i stor grad uberørt av både ekspansjonsbetingelsene og den opprinnelige cellekilden.

Målet med den tredje studien var å differensiere de genererte xenogenene og xeno-frie iPS til iMSC, og å vurdere og sammenligne deres osteogene potensial. Begge protokollene støttet differensiering av iPS til iMSC. Videre støttet protokollene osteogen differensiering av iMSC både *in vitro* og *in vivo*. Til slutt hadde den opprinnelige cellekilden liten innvirkning på den endelige iMSC geno/fenotype og osteogene potensial.

Denne avhandlingen har sett på fordelene og utfordringene som oppstår når man utleder iPS/iMSC fra ulike kilder under xeno-frie forhold. Samlet sett viser disse funnene vei for videre evaluering og undersøkelse.

List of Publications

This thesis is based on the following scientific papers. They will be referred to in the text accordingly.

Paper I: Salwa Suliman, **Hassan R.W. Ali**, Tommy A. Karlsen, Jerome Amiaud, Samih Mohamed-Ahmed, Pierre Layrolle, Daniela E. Costea, Jan E. Brinchmann, Kamal Mustafa. **Impact of humanized isolation and culture conditions on stemness and osteogenic potential of bone marrow derived mesenchymal stromal cells.** *Scientific Reports.* (2019) 9:16031

Paper II: **Hassan R.W. Ali**, Salwa Suliman, Tarig Al-Hadi Osman, Manuel Carrasco, Ove Bruland, Daniela-Elena Costea, Helge Ræder, Kamal Mustafa. **Xeno-free generation of human induced pluripotent stem cells from donor-matched fibroblasts isolated from dermal and oral tissues.** *Stem Cell Research and Therapy.* (2023) 14:199

Paper III: **Hassan R.W. Ali**, Salwa Suliman, Tarig Al-Hadi Osman, Daniela E. Costea, Shuntaro Yamada, Mohammed A. Yassin, Samih Mohamed-Ahmed, Cecilie Gjerde, Helge Ræder, Kamal Mustafa. **Osteogenic differentiation of induced pluripotent stem cells derived from dermal and oral tissues in xeno-free medium.** *(Submitted Manuscript)*

Abbreviations

AB	AB Serum
αMEM	Alpha Modified Essential Medium
AMSC	Adipose-derived Mesenchymal Stem Cells
ANOVA	Analysis of Variance
ALP	Alkaline Phosphatase
BCP	Biphasic Calcium Phosphate
BF	Buccal Fibroblasts
bFGF	Basic Fibroblast Growth Factor
BGLAP	Bone Gamma-carboxyglutamate Protein
BM-MSC	Bone Marrow-derived Mesenchymal Stem Cells
BMP	Bone Morphogenic Protein
βTCP	Beta Tricalcium Phosphate
BTE	Bone Tissue Engineering
CD	Cluster of Differentiation
COL1	Collagen Type 1
CNV	Copy Number Variation
CT	Computed Topography
D	Donor
DF	Dermal Fibroblasts

DMEM	Dulbecco's Modified Eagles Medium
DNA	Deoxyribonucleic Acid
ECM	Extracellular Matrix
ESC	Embryonic Stem Cells
FBS	Fetal Bovine Serum
FGF	Fibroblast Growth Factor
BF	Buccal Fibroblasts
GAPDH	Glyceraldehyde Phosphate Dehydrogenase
GATA4	GATA Binding Protein
GF	Gingival Fibroblasts
GMP	Good Manufacturing Practice
HA	Hydroxyapatite
HLA	Human Leukocyte Antigen
HOPX	HOP Homeobox
HS	Human Serum
IGF	Insulin-like Growth Factor
IL	Interleukin
iMSC	Induced Mesenchymal Stem Cells
iPS	Induced Pluritpotent Stem Cells
ISH	In Situ Hybridization

ISCT	International Society for Cellular Therapy
KLF4	Kruppel-like Factor 4
MESP1	Mesoderm Posterior BHLH Transcription Factor 1
MHC	Major Histocompatibility Complex
MSC	Mesenchymal Stem Cells
NANOG	Homeobox NANO G
NOD	Non-obese Diabetic
NSG	NOD SCID Gamma
OCT4	Octamer-binding Transcription Factor 4
PAX6	Paired Box 6
PDGF	Platelet-derived Growth Factor
PL	Platelet Lysate
PLATMC	Poly (L-lactide-co-trimethlene Carbonate)
PPARG	Peroxisome Proliferator Activated Receptor Gamma
PR	Platelet Releasate
qPCR	Quantitative Polymerase Chain Reaction
RAX	Retina and Anterior Neural Fold Homeobox
RNA	Ribonucleic Acid
RUNX2	Runt-related Transcription Factor 2
SCID	Severe Combined Immunodeficiency

SOX2   SRY Box Transcription Factor 2

SPP1   Secreted Phosphoprotein 1

TGFβ   Transforming Growth Factor Beta

TNFα   Tumor Necrosis Factor Alpha

VEGF   Vascular Endothelial Growth Factor

List of Figures and Tables

1. INTRODUCTION

1.1 BONE TISSUE ENGINEERING

Bone is a unique, dynamic, mineralized tissue which plays a major role in providing the human body with mechanical support [1]. Its complex environment comprises of cellular and extracellular components that work in tandem to develop the final bony structure [2, 3]. Bone tissue has the ability to completely regenerate without scar formation. This involves a distinctive healing process wherein bone independently undergoes repair, with the aid of surrounding tissue, and regains functionality [4]. In the event of a fracture, these molecules recruit blood vessels and undifferentiated mesenchymal stem cells (MSC) to the injury site. Continued signaling promotes MSC proliferation, and eventually differentiation into *RUNX2* expressing osteoblasts [2, 5]. Osteoblasts actively secrete extracellular matrix (ECM) rich in collagen type I, also known as osteoid, which subsequently mineralizes through the accumulation of hydroxyapatite. This mineralized ECM traps osteocytes, derived from differentiated osteoblasts, which form an extensive network with surrounding cells [5]. Osteoblasts and osteoclasts work simultaneously, via bone formation and resorption, respectively, leading to a balanced, continuous remodeling process. This balance is crucial for maintenance of bone structure, and a relative increase or decrease in osteoclastic/osteoblastic activity would ultimately compromise integrity [6, 7]. Bone's capacity for self-renewal, however, is not without limits. There are many instances in which the healing process might not succeed entirely, such as situations involving complex or pathological fractures, or instances of critical-sized defects where the defect is too substantial to overcome [8]. Various therapeutic strategies are currently being employed in attempts to address such complicated defects. The most common strategy typically entails transplanting autologous bone, harvested from the same individual, in the form of bone grafts. Besides reducing the risk of disease transmission, autologous grafts possess essential osteogenic, osteoinductive and osteoconductive properties necessary for inducing bone regeneration [9]. Despite being the gold standard to which other treatment strategies are compared, autologous bone grafts are

not suitable for relatively larger bone defects. Such cases require large amounts of bone that cannot be obtained from a local site. Furthermore, the harvesting procedure is invasive and may lead to surgery associated complications, including residual pain, hematoma formation, nerve injury, infection etc. [9, 10]. In attempt to avoid these limitations, allografts (bone from a different individual) and xenografts (bone from a different species) have been used. These substitutes come with disadvantages of their own, including the risk of transmitting viral and bacterial diseases [11, 12].

In light of the challenges associated with the available therapeutic strategies, scientists have searched for a more suitable alternative. This led to the development of a new approach to inducing bone regeneration, known as "Bone Tissue Engineering". Bone tissue engineering (BTE) induces the formation of new bone via combining osteoprogenitor cells and osteoinductive growth factors with a suitable biocompatible scaffold. These three components form the main 'triad' of tissue engineering and provide properties necessary for mimicking autologous bone, by restoring, maintaining and/or improving tissue function (**Figure 1A**) [13]. In further detail, BTE involves harvesting cells from an autologous source, followed by *in vitro* propagation until a desirable number of cells is obtained. Finally, the cells are seeded onto a suitable biocompatible scaffold, serving as a framework for tissue formation by enabling cell adherence and proliferation for *in vivo* application [13]. Scientists have also highlighted the importance of vascularization and mechanical stability, in the microenvironment of implanted scaffolds, to the success of the regeneration process (**Figure 1B**) [14, 15]. When appropriately combined, these components form an ideal environment for the formation of new bone.

Preclinical evaluation of proposed BTE strategies should ideally involve both *in vitro* and *in vivo* assessments. Typically, initial evaluations of the efficacy of any suggested therapeutic protocol being with *in vitro* studies [16]. Nevertheless, relying solely on these studies is insufficient for a direct progression to clinical trials, as they cannot accurately replicate intricate *in vivo* scenarios. As a result, pre-clinical *in vivo* animal models are necessary for the progression of these therapeutic strategies to human clinical trials [17, 18]. Preclinical *in vivo* evaluation generally comprises the use of

animal models to test novel BTE methods including small animals, such as rodents and rabbits, and larger animal models, such as dogs and minipigs [19]. Smaller models usually provide a platform for feasibility studies or proof of principle, while larger models are utilized in attempts to mimic clinical conditions in the human body [19]. A meta-analysis by Shanbhag et al., reviewing bone regeneration in preclinical settings, revealed BTE to be superior to cell-free scaffolds, while maintaining comparable results to autologous bone [19]. Although necessary for progression, results observed in preclinical studies do not always translate to clinical trials [20]. This further highlights the importance of clinical trials in the assessment of bone regeneration approaches. Since the inception of BTE, several clinical studies have been performed to assess the efficacy of different protocols [20-24]. The promising potential of BTE has also been demonstrated by our group, where a significant amount of bone formation was induced in mandibular bone defects, producing satisfactory esthetic and functional outcomes; as deemed by the patients involved [25].
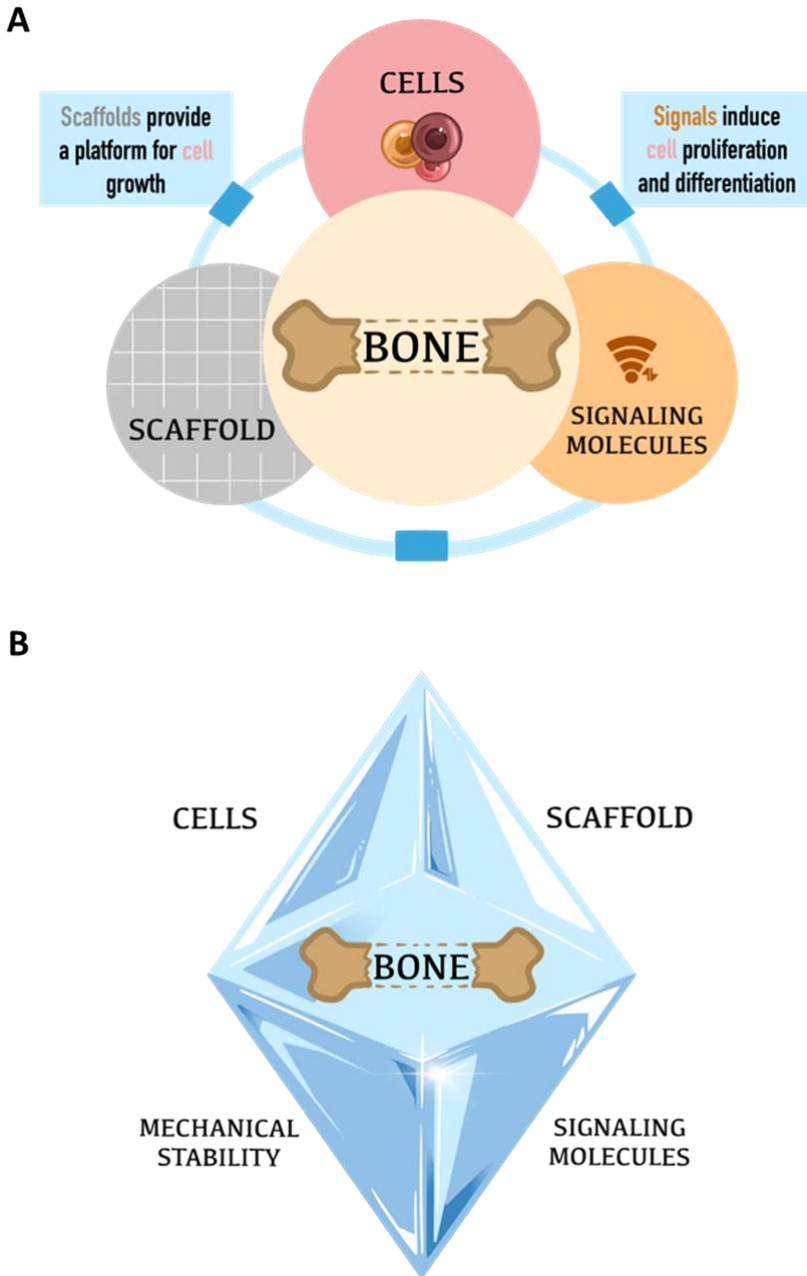
Figure 1. The *triad* of BTE and the *diamond concept* of bone regeneration

A) Diagram depicting the relationship between the *triad* of BTE. **(B)** Diagram depicting the *diamond concept* of bone regeneration. Figure adapted from Giannoudis et al [15]. This figure was created using Procreate 5.2 on iOS software.

1.2 SCAFFOLDS IN BONE TISSUE ENGINEERING

Scaffolds in BTE generally act as temporary, three-dimensional matrices, providing a platform conducive of tissue development and bone growth. They can be naturally or artificially derived, as synthetic graft substitutes, and can be with or without cells/drugs [26]. Specifically for bone regeneration, scaffolds should ideally be biocompatible, biodegradable, osteoinductive and conductive, should allow for cell adhesion and neovascularization, and promote cell growth [26, 27]. Calcium phosphate-based ceramics, such as biphasic calcium phosphate (BCP), are extensively used as BTE scaffolds. They gained popularity because of their bone-like properties and their similar composition to bone mineral [26, 28, 29]. Another key feature is their bioactivity, which enables them to interact with the surrounding environment. This interaction involves the release of calcium and phosphate ions, inducing osteogenic differentiation of stem cells [26]. BCP, a composite of hydroxyapatite (HA) and beta tricalcium phosphate (βTCP), combines their properties to form a relatively superior material. The degradation of BCP is contingent on the βTCP/HA ratio, with a higher ratio corresponding to increased degradation. This is attributed to the non-resorbable portion being formed by HA while βTCP consititues the bioresorbable part *i.e.*, a higher HA content in the composition results in reduced biodegradability of the material [28, 30]. BCP comprised of 80/20 ratio of βTCP/HA was found to have the greatest positive effect on bone formation [21, 29, 30].

1.3 SIGNALING MOLECULES IN BONE TISSUE ENGINEERING

Signaling molecules, in the form of hormones, cytokines and growth factors, represent one of the key elements for achieving bone regeneration. These molecules are secreted by cells and provide essential signals, in an autocrine, paracrine, or endocrine fashion, for new tissue formation [5]. Secreted signals induce the recruitment of blood vessels and undifferentiated MSC, and further promote cell proliferation and differentiation. Interleukins (*IL*), for example, are mainly pro-inflammatory cytokines involved in bone resorption and remodeling. Growth factors, on the other hand, influence bone

formation by regulating cell growth and function [2]. Bone morphogenetic proteins (BMP), member of the TGFβ superfamily, and basic fibroblast growth factor (FGF), member of the fibroblast growth factor (FGF) family, are examples that have been reported to demonstrate high osteoinductive properties and are strongly implicated in regulating the bone forming process [31-34]. Both BMP and FGF induce MSC differentiation towards the osteoprogenitor lineage [33-35]. BMPs also have an indirect influence by affecting the expression of other growth factors, which may lead to osteoblast differentiation and proliferation [33, 34]. FGF has also been reported to maintain the osteogenic potential of MSC during expansion [36]. The use of growth factors has also been implemented in clinical settings to achieve bone regeneration. For instance, in attempts to regenerate periodontal tissue, Kitamura et al. administered FGF into alveolar bone defects where they found a significant difference in rate of increase in bone height relative to the placebo [37]. Mesimäki et al. and Sándor et al. both combined BMP2 with MSC and a suitable scaffold to reconstruct critical sized defects in the maxilla and mandible, respectively, of adult patients [31, 32].

1.4 STEM CELLS IN BONE TISSUE ENGINEERING

1.4.1 EMBRYONIC STEM CELLS

Pluripotent cells exist naturally as embryonic stem cells (ESC) and have been extensively researched in the past for their regenerative and therapeutic potential [38]. ESC possess unique characteristics that separates them from other cells. Primarily, they are capable of indefinite self-renewal, can be expanded infinitely as they do not undergo replicative senescence. This may be attributed to their high telomerase expression. Telomerase is responsible for replacing the ends of shortened telomeres, thereby resetting the cells lifespan, and allowing for continued proliferation and cell division. Furthermore, one of the main features that separates ESC from other cells, is their pluripotent nature, *i.e.*, they can give rise to cells of endoderm, mesoderm, and ectoderm lineages (the three primary germ layers) [39, 40]. Such characteristics make ESC ideal for regenerative therapy. With time, however, red flags were raised concerning the methods in which they are isolated. ESC are isolated via unethical

means which mandate the destruction of embryos, a procedure which only yields a limited supply of cells. There are also major concerns regarding the outcome of allogenic transplantation of ESC due to their immunogenicity and accompanied risk of teratoma formation. They also express major histocompatibility complex (MHC) class I molecules which may ultimately lead to the rejection of ESC containing grafts [40, 41]. Despite the huge potential that was shown at the time, these hurdles led scientists to shift their focus elsewhere.

1.4.2 MESENCHYMAL STEM CELLS

Mesenchymal stem cells were first introduced by Friedenstein in the late 1960s, and since then have become a constant in the fields of stem cell research and regenerative medicine [42]. MSC are undifferentiated stem cells that possess multipotent and self-renewing capabilities [43]. These cells are easily extracted from various tissues, and their expansion *in vitro* is well established. What makes MSC invaluable however, is their multilineage differentiation potential. Under defined conditions, these cells can differentiate towards the mesenchymal lineages, specifically osteocyte, adipocyte, and chondrocyte lineages [44]. To standardize research surrounding MSC, the international society for stem cell therapy (ISCT) defined MSC according to the following set of criteria [45]:

- plastic adherence with a fibroblastoid phenotype
- cell surface expression of cluster of differentiation (CD) 73, CD90 and CD105 along with the lack of expression of CD45, CD34, CD14 or CD11b, CD79α or CD19 and human leukocyte antigen (HLA)-DR molecules
- *In vitro* differentiation capacity towards adipocyte, chondrocyte, and osteocyte lineages

Utilizing MSC in therapeutics requires formal approval by regulatory authorities. Manufacturing processes must comply with good manufacturing practice guidelines (GMP) to ensure the safety, purity, efficiency, and potency of proposed therapeutics. These guidelines address manufacturing issues and quality control measure, encompassing parameters such as donor selection, selection of culture media, and the

duration of expansion [46]. The production of MSC for cell therapy is a complex procedure, involving the acquirement of tissue, isolation of MSC, and finally the expansion of isolated cells. The final product must go through a number of quality control steps including microbiological tests, phenotyping to assess the degree of purity, as well as functional and safety testing [47, 48].

MSC possess many different "pro-regenerative" properties including, the ability to migrate to injury sites, potential to differentiate into different cell lineages, ability to release signaling molecules necessary for cell proliferation and survival, and modulation of the immune response and inflammation through the release of various anti-inflammatory and immunomodulatory factors [49]. They have been isolated and expanded from various sources, including bone marrow (BM-MSC), adipose tissue (AMSC), dental tissue, umbilical cord etc. [32, 50]. Of these sources, BM-MSC represent the most frequently used cells for bone regeneration purposes [51]. BM-MSC are readily obtained via bone marrow aspirates followed by isolation via adherence. The product is usually a heterogenous population of cells, as other cells adhere to the plastic surfaces as well, with BM-MSC comprising less than 1% of that population [52]. Therefore, *ex vivo* expansion strategies have been employed to enable scientists to reach the desired number of cells for pre-clinical and clinical purposes [49] [53]. These strategies, however, are impeded by the limited *in vitro* expansion potential of BM-MSC due to cellular senescence, which aggravates various functions, including proliferation and differentiation potential [54]. Hence, scientists are forced to utilize MSC at relatively earlier passages. Furthermore, MSC-use generally poses quite a few additional difficulties. First and foremost, current methods for collecting and ascertaining MSC from patients are expensive and require high expertise and infrastructure. Cell quality also tends to vary greatly, depending on a variety of factors including donor variation, source variation, isolation and culturing protocols etc. Such variation results in a heterogenous population of MSC exhibiting different phenotypes and functional properties [55, 56]. For instance, previous reports have shown a decrease in MSC quality with an increase in donor age [55, 57]. Zaim et al. compared MSC from young, adult, and elderly donors, and found that the cells developed irregular

morphology at passages 15, 9 and 5, respectively. They also reported a decrease in osteogenic and adipogenic differentiation potential of MSC from the older donors [57]. In attempts to battle the lack of standardization in MSC related projects, a standardized protocol was developed for the isolation and expansion of MSC for clinical purposes [24, 58]. Nevertheless, unless a standard protocol is universally implemented in all MSC related projects, there will always exist a degree of uncertainty surrounding data in this field.

1.4.3 INDUCED PLURIPOTENT STEM CELLS

In 2006, a scientific breakthrough was made when Shinya Yamanaka was able to genetically modify/reprogram adult somatic cells into cells with a similar gene expression profile and differentiation potential as ESC [59, 60]. These ESC-like cells were named 'induced pluripotent stem cells' (iPS). iPS typically resemble ESC in both geno- and phenotype. The similarity in characteristics include unlimited proliferation potential, expression of pluripotent markers, and the ability to give rise to the three primary germ layers, namely, endoderm, mesoderm, and ectoderm [61]. There are also no ethical concerns regarding the method in which iPS are generated, hence overcoming one of the major drawbacks of ESC use. Furthermore, iPS technology holds great promise for patient specific cell-based therapy, as autologously transplanted iPS avoid the risk of immune rejection, unlike allogenic ESC [61, 62]. The reprogramming process was first achieved via viral delivery of a cocktail of four transcription factors or 'Yamanaka Factors', namely OCT4, SOX2, KLF4 and c-MYC [59, 60]. Since then, various groups have successfully reprogrammed adult cells to iPS using different sets of transcription factors [63, 64]. A variety of different methods have been used to facilitate the introduction of reprogramming factors into the nucleus of somatic cells (**Figure 2**). These include both integrating and non-integrating methods. Integrating methods generally leave behind an undesirable footprint by integrating exogenous genetic material into the host genome, and include, among others, retro- and lentiviral methods. To make iPS more clinically translatable, non-integrating methods have been developed with zero footprint, and include the use of adenoviral, episomal plasmids and protein delivery methods [41, 64, 65]. Despite reports on the spontaneous

loss of episomal plasmids following cell transfection, [64, 66], other footprints may still occur, including artificially introduced copy number variations (CNV). CNVs such as deletions and duplications are natural structural variants within the human genome. Such aberrations have been regularly reported in the study of human pluripotent cells, with iPS generally having a higher number of CNVs than ESC [67-69]. These are largely due to the initial transfection procedure, as generated iPS are associated with a high number of CNVs in the early stages/passages. The total size and number of these CNVs, however, decreases dramatically as the propagation/culture of iPS continues [70]. In addition to providing the unique qualities of ESC, somatic cell reprogramming has the potential to provide an unlimited source of pluripotent stem cells. While isolating a sufficient amount of ESC would require the destruction of a large number of embryos, an unlimited supply of iPS can be generated from reprogramming adult somatic cells. Other advantages of iPS technology include disease modelling, drug screening and gene targeting/editing technology [41, 61].
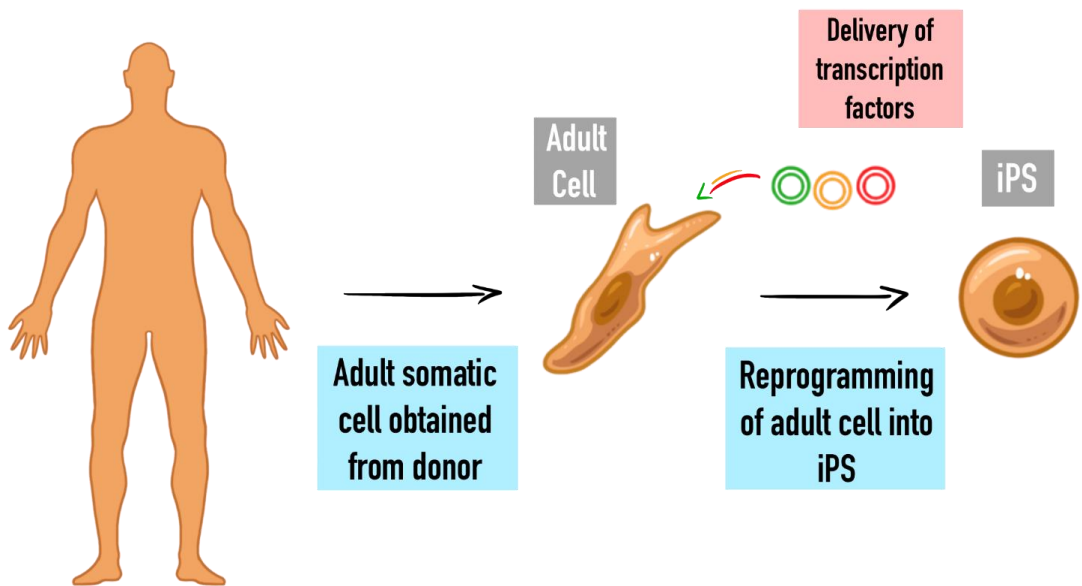
Figure 2. Illustration of somatic cell reprogramming into iPS. This figure was created using Procreate 5.2 on iOS software.

1.4.3.1 SOURCES OF IPS

The various reprogramming methods mentioned above have been utilized to generate iPS from a number of different sources, including fibroblasts, as the most common source, keratinocytes, blood cells, dental pulp stem cells etc. [71-75].

FIBROBLASTS

Fibroblasts have long been deemed as a favorable source for iPS generation due to the relative ease in which they are cultured/expanded. In fact, the first batch of iPS were generated from murine dermal fibroblasts by Yamanaka and co [59]. The same group then followed that up by reprogramming human dermal fibroblasts into iPS [60]. Since then, various groups have generated iPS from different fibroblast sources. Following the groundbreaking discovery, dermal fibroblasts have been the most commonly reprogrammed group, as they can be easily obtained through relatively simple surgical procedures [72]. There does, however, exist a risk of scar formation when fibroblasts are surgically obtained from the dermis. Alternatively, the use of oral fibroblasts for iPS generation has reduced the risk of long term scar formation [76]. Furthermore, these cells can be easily obtained during routine oral procedures from the gingiva, buccal mucosa, and periodontal ligament [73, 74]. Coupled with rapid healing following biopsy collection, oral fibroblasts might present as an excellent alternative to dermal fibroblasts [73, 76]. Nevertheless, one major drawback of fibroblasts collection, whether dermal or oral, is that surgical procedures require selected areas to be anesthetized prior.

BLOOD CELLS

As blood can easily be accessed and is abundantly found in the body, it has been considered as a quality source of cells for reprogramming purposes [72, 77]. Peripheral blood cells have been harvested using mobilized CD34+ cells. Briefly, in the presence of G-CSF, hematopoietic stem cells are mobilized from the bone marrow into the peripheral blood. At this stage, the CD34+ cells can be separated and isolated from blood via aphaeresis. This method, however, is invasive and time consuming, and may present with aftereffects such as fatigue, headaches, nausea etc. (related to the injection

of G-CSF) [72]. Alternatively, specific mononuclear cells can be isolated by density gradient centrifugation, a labor intensive and technique sensitive method [72, 78]. Higher reprogramming efficiencies have also been reported, where a single blood drop was sufficient for cell isolation and subsequent reprogramming [77].

OTHER

Keratinocytes, which can be isolated from skin, hair, and nails, have also been successfully reprogrammed into iPS. Previous groups have generated iPS from foreskin and hair follicle keratinocytes with high efficiency [79, 80]. Aasen et al showed a higher efficiency when reprogramming keratinocytes compared to fibroblasts, as well as a much faster rate of colony formation (6-7 days post transfection)[79]. Although demonstrating higher reprogramming efficiencies, dermal keratinocytes, like dermal fibroblasts, are obtained from the dermis. This means that the administration of an anesthetic prior to surgical collection is required. Moreover, the surgical procedure results in scar formation. On the other hand, keratinocytes can also be obtained from hair follicles [72, 81]. Hair follicles as a source of keratinocytes presents the advantage of convenience, as they can simply be plucked from donors. Nevertheless, generating a sufficient number of cells from hair follicles requires high expertise in the isolation and culture of keratinocytes. Furthermore, hair follicle keratinocytes have been shown to undergo senescence relatively early i.e., keratinocytes from early passages are preferably used for iPS generation [72]. Different types of cells have also been isolated from urine samples and subsequently reprogrammed into iPS, namely epithelial cells and urine-derived stem cells [82-84]. The collection procedure is typically noninvasive, simply requiring a urine sample from the patient/donor [83, 84]. Hence, since urine sample collection is relatively simple, cheap, and noninvasive, some might argue that urine cells present as an ideal source for iPS generation.

1.4.3.2 LIMITATIONS AND CONCERNS SURROUNDING IPS

Despite the many benefits of iPS technology, scientists are still faced with many challenges. Primarily, as mentioned above, some delivery methods result in the integration of exogenous genetic material into the host genome. This can be avoided,

however, by simply utilizing a non-integrating method for reprogramming factor delivery [41, 64, 65]. Another concern surrounding iPS is the associated risk of tumor formation, with the transcription factor c-Myc in particular being a known oncogene [85, 86]. To avoid this oncogenic property, multiple protocols have since replaced c-Myc with another, less tumorigenic, member of the MYC family, namely L-Myc [63, 64], while others have removed MYC altogether [87]. One major limitation of iPS technology is the difficulty surrounding the induction of pluripotency. Inducing pluripotency in somatic cells is a very inefficient process, with efficiency levels as low as 0.0006% [88]. Different theories have been postulated to explain the reason for this low efficiency, namely, elite, stochastic and deterministic models. Prior to explaining the reasons for this failure and low efficiency, one must understand the reprogramming process itself. Based on previous reports, the reprogramming process generally consists of two phases, a primary stochastic phase, and a secondary more deterministic phase [41]. Briefly, the primary phase usually involves (1) the down regulation of lineage specific genes, (2) the activation of ESC genes and (3) chromatin remodeling at pluripotency genes sites by the unfolding of condensed chromatin and the removal of repressive chromatin marks. The secondary phase involves (1) the restoration of the ESC transcription network, characterized by telomerase activity and ESC signaling cascades, and (2) transgene silencing. Thus, there are many events that must be completed for a cell to be reprogrammed. If a cell fails to complete these events before complete transgene silencing, it will revert to its differentiated state. Completion of both phases of reprogramming appears to be a rare event for most cells, hence the low reprogramming efficiency levels [41]. There are also concerns regarding the immunogenicity of iPS and their derivatives, despite conflicting reports, as some studies have reported that they can be targeted by both allogenic and autologous immune systems [89, 90]. Moreover, iPS related research and therapy requires a relatively high budget, as producing and characterizing each individual iPS cell line is quite costly [41]. Currently, iPS are mainly used as tools to better understand disease mechanisms. A few limitations make it extremely difficult to take full advantage of their high regenerative abilities [61]. One major concern is iPS instability. iPS must be thoroughly tested prior to their use. This would entail relatively long culturing periods

which can lead to the development of karyotypic anomalies and copy number variations. iPS also have the potential to randomly differentiate into teratomas when implanted *in vivo* [91]. Such characteristics greatly limit the clinical use of iPS and therefore most clinical uses of iPS encompass their differentiated by products. This section will mainly be dedicated to the byproducts of differentiated iPS.

Although not directly, teratoma formation is potentially another area of concern that plagues the differentiated products of iPS. Prior to any clinical trials, it is vital to ensure that there are no undifferentiated cells in the final product that may undergo any unwanted transformation. Hence, current protocols need to be vigorously tested, improved, and optimized in animal models before a transition to human trials. The first clinical trial using iPS started in 2014 specifically for the treatment of macular degeneration [92, 93]. Two patients were transplanted with autologous iPS derived retinal pigment epithelial cells. In the first patient, the degeneration was halted and improved vision was achieved. However, the trial on the second patient was placed on hold due to the discovery of genetic aberrations within the iPS [92]. Of specific clinical importance is the immunogenic nature of iPS and their end products. Currently, studies are in conflict with some showing low immunogenicity of iPS products, while others highlight a more immunogenic nature [89, 90, 94-96]. Moreover, some studies have demonstrated that even autologous iPS products can trigger an immune response due to their genomic instability [91].

1.4.3.3 iPS-DERIVED MESENCHYMAL STEM CELLS

MSC-like cells have been successfully derived from iPS (iMSC) and have been shown to express MSC surface markers, exhibit plastic adherence, and the ability to differentiate towards osteogenic, adipogenic and chondrogenic lineages. Several differentiation protocols have been employed for differentiating iPS to iMSC. The protocols typically involve either the generation of embryoid bodies (EB) prior to differentiation, or the direct differentiation of iPS to iMSC. Following EB formation, cells emerging from these bodies are usually propagated until, eventually, cells with a homogenous fibroblastic morphology appear [97]. On the other hand, the direct differentiation of iPS to iMSC skips the EB formation step and cells with a fibroblastic

morphology emerge directly from iPS colonies. Similarly, the emerging cells are further propagated until a homogenous group of morphologically similar cells are obtained [98, 99]. Regardless of the protocol selected, the strategy generally involves depriving culturing media of pluripotent signals, usually achieved by replacing iPS culturing media. This leads to spontaneous differentiation of iPS into cells with a fibroblast-like appearance. Once these cells satisfy the criteria set by the ISCT (see above), they are considered as iMSC. As iPS can be expanded indefinitely, they present as a potentially inexhaustible source of large amounts of iMSC [97-100].

As mentioned previously, naturally isolated MSC present with quite a few drawbacks which highlight the value of alternative options such as iMSC. These include difficulties in obtaining large amounts of BM-MSC through aspiration alone, as these cells comprise approximately less than 1% of the aspirated population, requiring further *ex-vivo* expansion to reach desired amounts. Coupled together with early senescence, there is very little room for prolonged/further expansion prior to utilizing the cells for scientific purposes [49] [53]. iMSC on the other hand, have shown a much greater capacity for proliferation and expansion, with the ability to expand to approximately 40 passages without an obvious onset of replicative senescence [101]. This allows for more flexibility since it is not necessary, unlike for regular MSC, to utilize cells at an early stage.

1.5   CELL EXPANSION CONDITIONS: CONCERNS AND ALTERNATIVES

1.5.1 FETAL BOVINE SERUM

As previously highlighted, the manufacture of MSC for cell therapy must be compliant with current GMP guidelines [46]. As of now, fetal bovine serum (FBS), is the most commonly used supplement to MSC expansion medium [102]. It provides basic factors and hormones required for the stimulation of cell growth/proliferation and provides detoxifying factors which inhibit the action of toxic molecules. FBS is also favored because of its relatively low gamma-globulin content, as high antibody content may decrease cell growth/proliferation [103]. Despite being the most common supplement,

the use of FBS for clinical applications is linked to a multitude of ethical, scientific and safety concerns. Primarily, obtaining FBS is considered to be an inhumane form of extraction, as fetal blood is collected via cardiac puncture of the unanesthetized fetus. Furthermore, the composition of FBS varies from batch to batch, affecting its reproducibility and its use in scientific experiments. Major safety concerns have also been raised due to the xenogenic origin of FBS, as it is potentially a source of xenogenic immune reactions and bovine pathogen transmission [104, 105]. Hence, the clinical use of FBS has been prohibited in an increasing number of countries. As a result, FBS production has come under great scrutiny and regulatory authorities have discouraged the use of animal-based sera for *ex vivo* expansion of MSC [106, 107]. There has since been a growing demand for animal-free culture conditions, which would allow for safer and more ethical practices.

1.5.2 XENO-FREE CONDITIONS

Alternative protocols have been developed with the aim of establishing xeno-free conditions for MSC culture. These primarily involve two major categories of media supplements, namely human derived, which includes human serum (HS) (autologous or allogenic) and platelet derivatives (platelet lysate and platelet releasate), and chemically defined media [108, 109]. Human derived products naturally contain high concentrations of growth factors and cytokines making them promising alternatives to conventional FBS.

1.5.2.1 HUMAN SERUM

The effect of HS on MSC expansion is well documented, as numerous studies have compared HS culture conditions to FBS [110-112]. Studies have shown that 10% autologous HS is at the very least comparable to FBS in MSC isolation and expansion, while lower concentrations proved to be less efficient [110, 113]. In some studies, however, differentiation potential was found to be diminished in autologous HS expanded MSC as opposed to those expanded in FBS [110]. Despite such promising findings, the limited availability of autologous HS presents a major challenge, as large amounts of autologous blood are required for long term MSC expansion [19]. In order to overcome this issue, scientists have investigated the use of allogenic HS as a

supplement to MSC expansion medium, commonly in the form of human AB serum (AB)[114, 115]. Although AB has been proven to support the proliferative and differentiation capacity of MSC, there are many conflicting reports regarding its use as an MSC expansion supplement [115, 116]. These discrepancies in reports may be due batch-to-batch variation in the serum employed [117]. In some cases, growth factors, such as basic fibroblast growth factor (FGF), are commonly added to culture medium to positively influence cell expansion by enhancing proliferation and differentiation [118, 119]. Most of the data demonstrates that AB is a suitable alternative for FBS in supporting the growth and biological functions of MSC. Nevertheless, there are still a few challenges that ultimately need to be tackled. Primarily, when using allogenic serum, there exists a risk of human pathogen transmission. Furthermore, donor-age related changes in serum may lead to variations in MSC behavior [120]. Due to such drawbacks, platelet derivatives have emerged as a practical alternative to FBS for long term clinical grade expansion of MSC.

1.5.2.2 PLATELET LYSATE

Platelet concentrates are extensively utilized in regenerative therapy to administer growth factors at relatively high concentration levels. Platelets generally play a major role in the wound healing process. A role which involves the release of high concentrations of signaling molecules, including PDGF, FGF, IGF, VEGF and others [121, 122]. It is this natural release of growth factors that makes the use of platelet derivatives so promising in cell culturing methods. These molecules, released mainly from platelet α granules, support cell expansion, function, and proliferation [122, 123]. Platelet derivatives are generally used in an autologous form, or as pooled products, *i.e*., pooled from multiple donors. The use of autologous platelet derivatives eliminates the risk of disease transmission. Nonetheless, as is the case with autologous HS, obtaining a sufficient amount is generally a challenge, especially when donors are not eligible for multiple blood donations. On the other hand, pooled platelets generally provide large quantities of platelet products while also reducing donor-based variations in platelet count and growth factor content [19]. Pooled platelet products can also be manufactured from expired platelet concentrates which are usually discarded by blood

banks after 4-5 days as they are unsuitable for transfusion. Hence, using expired platelet concentrates offers a notable advantage over using fresh platelet concentrates [19, 124].

The preparation of platelet lysate (PL) typically involves isolating platelet rich plasma from the buffy coat or platelet apheresis, after which it is subjected to either mechanical disruption or physiological/chemical activation (**Figure 3**). During the mechanical method, platelets undergo repeated freeze/thaw cycles forming ice crystals which disrupt the platelets membrane. An alternative method is achieved through either sonication alone, or sonication in combination with freeze/thaw cycles. The chemical activation method involves the addition of calcium chloride, collagen, or bovine thrombin to isolated platelets in order to obtain the lysate. The resultant lysate obtained via chemical activation is termed platelet releasate [125]. The disruption method is generally preferred over the activation method due to its simplicity and cost effectiveness, however studies have shown differing proliferation rates of MSC grown in PR when compared to those grown in PL [19].
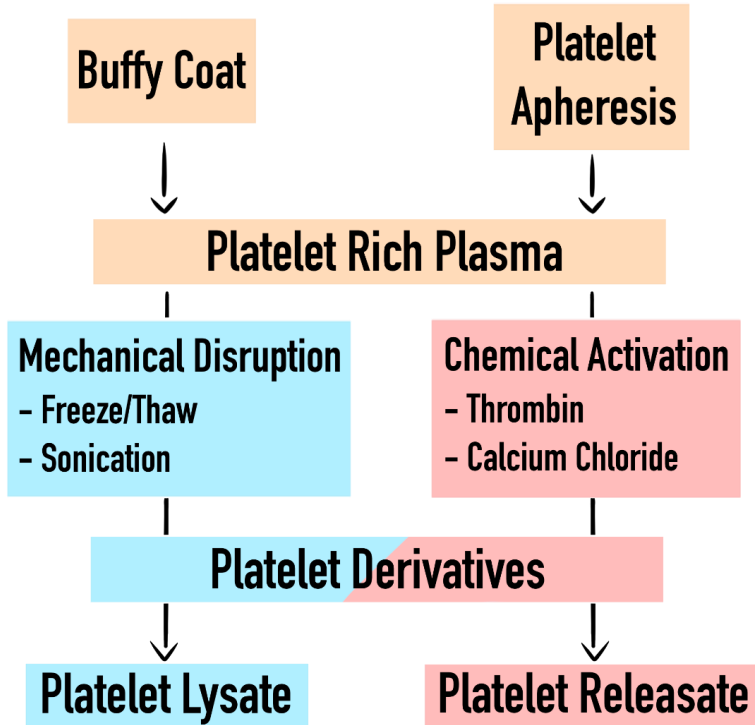
Figure 3. Steps involved in the preparation of lysate from platelet rich plasma. This figure was created using Procreate 5.2 on iOS software.

Human PL was first suggested as an alternative to animal serum for the expansion of MSC in 2005, where Doucet et al. demonstrated that medium supplemented with PL promoted expansion and proliferation more efficiently than FBS supplemented medium. [126]. Since then, its popularity has grown substantially, with an increasing number of studies and clinical trials using PL for *in vitro* cell expansion [25, 109, 114, 127-129]. In addition, multiple studies have reported that PL enhances the proliferation and expansion of MSC in comparison to FBS, further supporting the results obtained by Doucet et al [126, 130-132]. The literature has also reported the ability of PL to support, and in many cases enhance, osteogenic differentiation of MSC *in vitro* [129, 133-135]. Nevertheless, conflicting reports do exist, most likely arising from either the

different concentrations of supplements used, batch variability and type of cell, or a combination of these factors [136].

2. RATIONALE

An iMSC-based approach for BTE purposes has emerged as a viable alternative to the use of BM-MSC. A major advantage of this approach is that iMSC can be obtained from different types of iPS *i.e.*, iPS generated from different sources [97-99]. Nevertheless, as the reprogramming of cells is a tedious process, it is imperative to determine optimal sources for high quality iPS that are relatively easier to reprogram. Various sources have been used and compared for iPS generation [71-75], however, unless donor-matched, the comparison is ultimately subpar, as the findings are more than likely influenced by donor variation.

To facilitate clinical translation, it is absolutely vital that cell culturing protocols for BTE are GMP compliant and free of xenogenic elements [46]. It is equally important that these protocols are thoroughly assessed, and their overall efficiency/safety is compared to gold standard protocols. Hence, iPS intended for future BTE purposes must be generated under xeno-free conditions. However, such studies revolving around xeno-free iPS production are scarce [137-140]. These novel xeno-free approaches must be ultimately compared to established, xenogenic protocols.

The literature shows a plethora of studies involving xeno-free strategies for BM-MSC expansion. To our knowledge however, only a handful of studies have generated and cultured iMSC from xeno-free conditions [141]. In most cases, these studies fail to mention whether the expansion conditions were entirely xeno-free, *i.e.*, the iPS used for iMSC generation and the parent cells used for iPS generation were expanded in xeno-free conditions. Also, no effort was made among these studies to compare iMSC for BTE generated via xeno-free protocols to those generated via xenogenic protocols, which is an essential prerequisite.

3. AIMS

The overall objective of this project was to develop a xeno-free protocol for the generation of iMSC and to determine their osteogenic capacity. A sub-objective was to investigate several donor-matched sources for the generation of iPS and subsequently, iMSC.

In the context of this thesis, the specific aims for each individual paper are as follows:

1. To identify an appropriate xeno-free protocol for human BM-MSC expansion, and investigate its effect on their osteogenic potential, by evaluating two separate xeno-free strategies. (***Paper I***)

2. To determine reprogramming efficiency and characterize ensuant donor-matched human iPS generated via xeno-free strategies compared to those generated via xenogenic protocols. (***Paper II***)

3. To investigate xeno-free and xenogenic iMSC generated from the iPS in ***Paper II***, and to determine their osteogenic potential via *in vitro* and *in vivo* methods. (***Paper III***)

4. MATERIALS AND METHODS

This section primarily contains a summary of methodology implemented in this thesis, with a more detailed description available in the attached manuscripts.

4.1 CHOICE OF METHODS AND STUDY DESIGN

The ability to generate iMSC from iPS through xeno-free strategies can potentially pave a new path for bone regeneration. However, as with any novel approach, pre-clinical studies must be performed to evaluate its safety, efficacy, and osteogenic potential. A selection of *in vitro* and *in vivo* methods was implemented based on a thorough review of the literature. The methods used in this thesis are summarized in **Figure 4**.
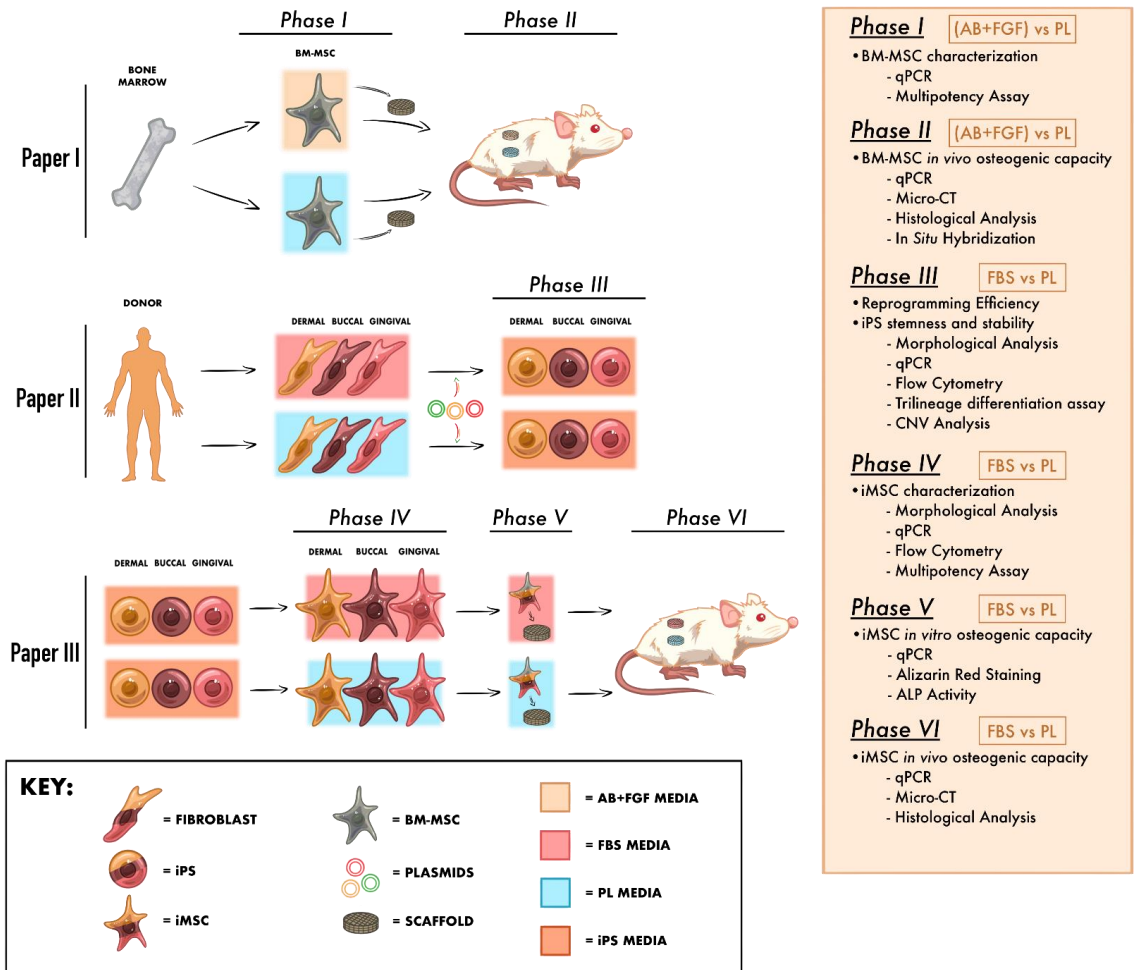
Figure 4. Thesis study design. This figure was created using Procreate 5.2 on iOS software.

In the interest of clinical translation, this thesis focuses on the effect of using humanized culture conditions for cell expansion, maintenance, etc. The majority of studies in this area involve comparing xeno-free protocols to the standard FBS utilizing protocol [126, 129, 133]. Instead, we decided to take a different approach and compare two xeno-free protocols in hopes of identifying an efficient substitute to generic FBS protocols for MSC expansion. In *paper I,* the focus was on assessing and comparing the impact of two separate xeno-free isolation/culture conditions, namely human PL

and AB supplemented conditions, on the stemness and osteogenic potential of BM-MSC. Cell stemness was determined via an *in vitro* assessment of multilineage potential, inflammatory response, and replicative senescence via quantitative real-time polymerase chain reaction (qPCR) analysis and flow cytometric analysis of specific surface markers. The *in vivo* osteogenic potential and inflammatory response of the cells was assessed following subcutaneous implantation of cell/scaffold constructs in non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice. The cell/scaffold construct was prepared by seeding the cells on biphasic calcium phosphate (BCP) granules. Samples were then collected at two separate time points, 1 and 11 weeks, after which qPCR was performed to measure the expression of certain inflammatory and osteogenic markers. In addition, histological analysis and histomorphometry were performed to assess the amount and frequency of bone formation. In *situ* hybridization was also performed to detect the presence or absence of human cells within the collected samples.

Obtaining iPS from somatic cells may be a long and tedious process. Therefore, matched dermal and oral (buccal and gingival) fibroblasts were reprogrammed in attempts to compare and develop an efficient xeno-free reprogramming protocol (***paper II***), and ultimately a protocol for generating iMSC (***paper III***) (**Figure 5**). The protocol involved culturing and expanding fibroblasts/iMSC in PL supplemented medium, which was compared to a protocol utilizing FBS supplemented medium (gold standard). To achieve cellular reprogramming, we utilized a non-integrating, episomal plasmid, delivery method to introduce a set of reprogramming factors into the nucleus of previously isolated fibroblasts. This particular method was selected as it ensures the absence of any undesired footprints in the host cell genome [41]. Once cell reprogramming is achieved, it is imperative to confirm the stemness and safety of the reprogrammed cells. Hence, the pluripotency of the generated iPS was assessed at a molecular, cellular, and functional level through various analyses (**Table 1**). Karyotyping was also performed to determine iPS safety and stability (***paper II***). The iPS were then differentiated to iMSC, by replacing iPS culturing medium with PL or FBS supplemented medium, thereby depriving cells of pluripotent signals. Their MSC-like status was then evaluated through observation of morphological changes, flow

cytometrical analysis of specific MSC markers, and multilineage potential analysis (osteogenic, adipogenic and chondrogenic lineages).

Both *in vitro* and *in vivo* methods were employed to assess the osteogenic potential of generated iMSC (***paper III***). Primarily, for *in vitro* assessment, iMSC were seeded onto poly (L-lactide-co-trimethylene carbonate) (PLATMC) scaffolds and osteogenically induced for 1- and 3-week time points. The assessment included measuring cell proliferation, expression of early/late osteogenic markers, alkaline phosphatase (ALP) activity, and amount of extracellular calcium deposition. For *in vivo* assessment, iMSC/PLATMC constructs were osteogenically induced for 7 days before being implanted subcutaneously on the dorsum of NOD/SCID-gamma (NSG) mice for 8 weeks. Samples were then collected and assessed for the expression of osteogenic markers by qPCR, and for the amount of mineralization by micro-CT and histological analysis. In *situ* hybridization was also performed to detect the presence or absence of human cells within the collected samples.
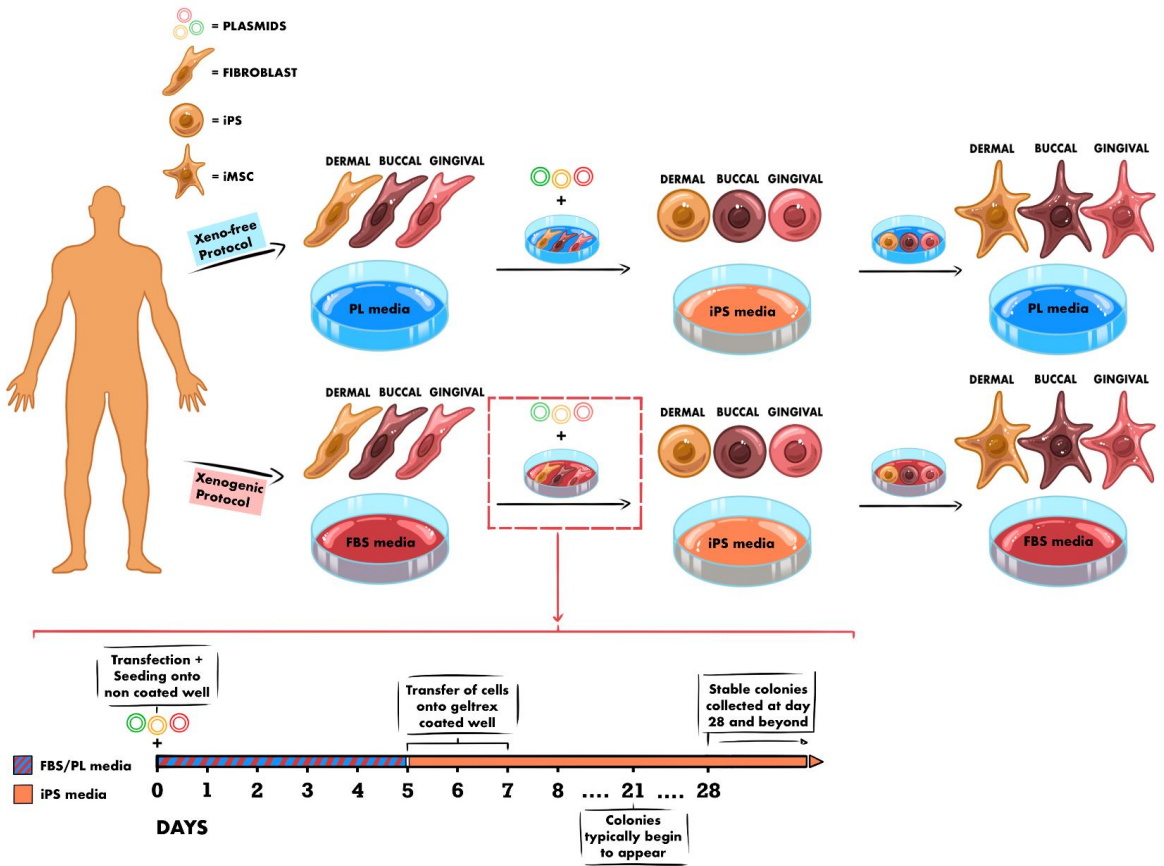
Figure 5. Generation of xeno/xeno-free iPS

Diagram illustrating the xeno/xeno-free generation of iPS, from different sources of matched fibroblasts, and their subsequent differentiation to iMSC. This figure was created using Procreate 5.2 on iOS software.

Table 1. Methods implemented for the evaluation of iPS.

	Methods
Molecular Level	Gene expression analysis: qPCR

	CNV analysis
Cellular Level	Morphological analysis
	Flow cytometry
Functional Level	Trilineage differentiation analysis
	Differentiation to iMSC

4.2   ETHICAL APPROVAL

Approval was granted by the regional Committees for Medical and Health Related Research Ethics in Norway: REK S-07043a (BM-MSC in *paper I*), REK 7199 sør-øst C (BM-MSC in *paper II*) and REK 80005 (fibroblasts, iPS and iMSC in *paper II* and *III*). Tissues were harvested from voluntary donors after acquiring informed and/or written consent. All animal experiments were approved by the Norwegian Animal Research Authority and conducted in strict accordance with the European convention for the protection of Vertebrates used for Scientific Purposes: FOTS No. 7894 (*paper I*) and FOTS No. 18738 (*paper III*).

4.3   CELL ISOLATION AND CULTURE (All Papers)

Several cell types were utilized for experiments throughout this thesis, and various conditions were employed for cell expansion. A description of cell expansion conditions for each cell type can be found in **table 2**.

4.3.1 Bone Marrow Mesenchymal Stem Cells (*Paper I* and *III*)

In *paper I*, BM-MSC were isolated from the bone marrow of three healthy donors after written informed consent. Following isolation, the cells were characterized and assessed using different bone marrow processing and expansion culture conditions. A detailed description of these conditions for each donor can be found in *paper I*. Briefly, BM-MSC were cultured separately in alpha modified essential medium (αMEM, Gibco) supplemented with either 10% human AB + 10 ng/ml FGF (Sigma-Aldrich) (AB-FGF), or 10% PL (Blood bank, Haukeland hospital, Bergen, Norway) (PL medium) and 1% Penicillin/Streptomycin (GE, Healthcare). Heparin (LEO Pharma) was added to PL supplemented medium at a concentration of 2 IU/ml to prevent gelation.

In *paper III*, BM-MSC served as controls for *in vitro* and *in vivo* osteogenic potential experiments. They were cultured separately in αMEM supplemented with either 10% FBS (FBS medium) or 5%PL (Blood bank, Haukeland hospital, Bergen, Norway) and 1% Penicillin/Streptomycin. Heparin was added to PL supplemented medium at a concentration of 2 IU/ml to prevent gelation.

4.3.2 Fibroblasts (*Paper II*)

Donor-matched oral (buccal (BF) and gingival (GF)) and dermal (DF) samples were acquired from two healthy voluntary donors (D1 and D2), after which the samples were processed, and fibroblasts isolated. All samples were collected from each donor; dermal samples were obtained from the anterior forearm, buccal samples from the inside of the cheek, and gingival samples from the gingiva above the upper first molar. Briefly, the cells were isolated via the enzymatic digestion protocol as previously described [142]. Following isolation, the fibroblasts were cultured and expanded separately in Dulbecco's Modified Eagles Medium (DMEM, Gibco) supplemented with 10% FBS or 5% PL, creating two different culture conditions (groups) for fibroblast expansion. Heparin was added to PL supplemented DMEM at a concentration of 2 IU/ml to prevent gelation.

4.3.3 Induced Pluripotent Stem Cells (generation and culture) (*Paper II*)

DF, BF and GF from each group (PL and FBS) were reprogrammed separately (Amaxa Nucleofector Kit, Lonza) to generate iPS. Following transfection, the cells were plated onto a six well plate containing either FBS or PL supplemented DMEM (**Figure 6A**). Upon reaching confluency, at approximately 7 days post transfection, cells were passaged onto a 10 cm dish pre-coated with Geltrex (Gibco). The following day, the medium was changed to StemFlex medium (StemFlex Medium, Gibco) supplemented with 1% Penicillin/Streptomycin. The medium was then changed every 1-2 days until colonies began to appear. With the help of a digital microscope (DMS1000, Leica Microsystems, Germany) newly formed colonies were transferred to a Geltrex coated well in a 24 well plate, one colony per well. Each iPS colony was cultured individually in StemFlex medium (**Figure 6B**). Three individual colonies, considered as biological replicas, were then chosen from each sample (both groups) for further evaluation.
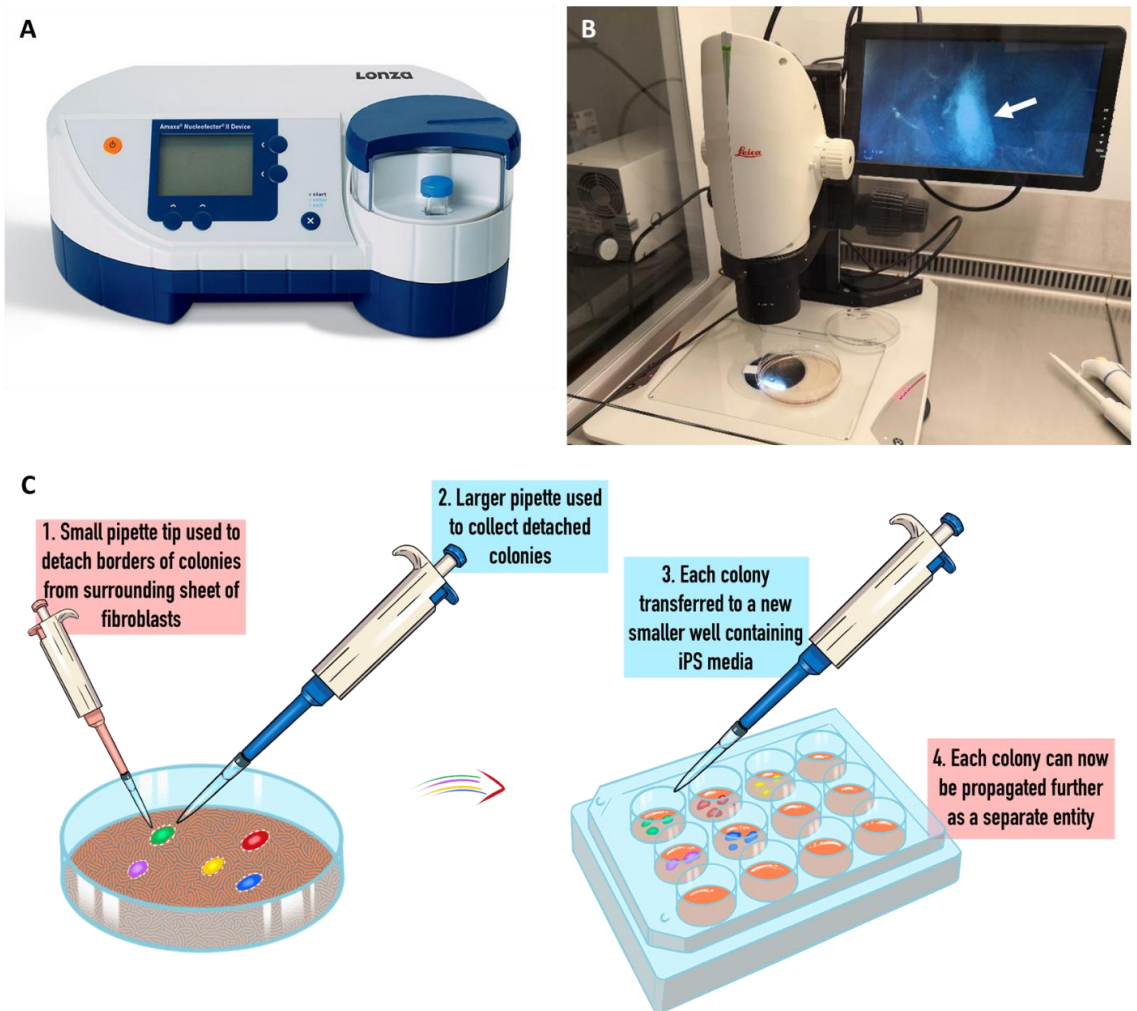
Figure 6. Collection of newly formed iPS colonies

A) The Amaxa Nucleofector 2b device used for cell transfection (image obtained from the Bioscience/Lonza official website). **B)** Leica digital microscope used to collect newly formed colonies. Arrow pointing to a clearly demarcated colony prior to collection. **C)** Diagram illustrating the collection of newly formed colonies from transfected fibroblasts; each individual colony is transferred to a new well and subsequently propagated. This illustration was created using Procreate 5.2 on iOS software.

4.3.4 Induced Mesenchymal Stem Cells (generation and culture) (*Paper III*)

The iPS were dissociated and plated onto new geltrex coated wells at a ratio of 1:10 containing StemFlex medium. Once the iPS proliferated and formed large compact colonies, medium was changed to αMEM supplemented with either 10% FBS or 5% PL, as previously described for BM-MSC. The medium was changed every 3 days and an outgrowth of cells from the colonies could gradually be observed. Once confluent, the differentiating cells were passaged at a ratio of 1:5 and plated onto non coated wells. The medium was changed every 3 days and subsequently passaged at a ratio of 1:10. This process continued until the cells appeared to show a fibroblast-like morphology. From that point, passage 4 onwards, the term iMSC was used to describe the cells. The generated iMSC were maintained separately in αMEM supplemented with 10% FBS or 5% PL, as previously described for BM-MSC, creating two different culture conditions (groups) for iMSC expansion (**Table 3**).

Table 2. Summary of cell culture conditions.

Cell type		Cell Culture Conditions
BM-MSC	*Paper I*	αMEM + 10% AB + FGF + 1% Penicillin/Streptomycin
		αMEM + 10% PL + 1% Penicillin/Streptomycin + Heparin
	Paper III	αMEM + 10% FBS + 1% Penicillin/Streptomycin
		αMEM + 5% PL + 1% Penicillin/Streptomycin + Heparin
Fibroblasts		DMEM + 10% FBS + 1% Penicillin/Streptomycin
		DMEM + 5% PL + 1% Penicillin/Streptomycin + Heparin
iMSC		αMEM + 10% FBS + 1% Penicillin/Streptomycin
		αMEM + 5% PL + 1% Penicillin/Streptomycin + Heparin
iPS		StemFlex + 1% Penicillin/Streptomycin

4.4   SCAFFOLD MATERIAL AND FABRICATION

In *paper I*, BCP granules composed of βTCP and HA, at a ratio of 80/20 by weight, were selected as the scaffolding material of choice. BCP granules ranging in size from 1-2 mm were supplied by Biomatlante (MBCP+, Vigneux de Bretagne, France).

In *paper III*, PLATMC scaffolds, prepared via a salt leaching technique [143, 144], were selected as the scaffolding material of choice. For more information on the selection and fabrication of scaffolding material, refer to *papers I* and *III*.

4.5 *IN VITRO* ANALYSES

Several *in vitro* methods have been employed in this thesis, and a detailed description of each can be found in their respective manuscripts. A summary of *in vitro* and *in vivo* methods used in this thesis can be found in **table 3**.

Table 3. Summary of *in vitro* and *in vivo* methodology.

Methodology	*In vitro / In vivo*	Paper
Alkaline phosphatase assay	*In vitro*	*Paper III*
Cell isolation	*In vitro*	*Paper I* and *II*
Cell culture	*In vitro*	All Papers
Cell differentiation	*In vitro*	*Paper III*
Cell reprogramming	*In vitro*	*Paper II*
Chromosome microarray analysis	*In vitro*	*Paper II*
DNA quantification assay	*In vitro*	*Paper I* and *III*
Flow cytometry	*In vitro*	All Papers
Gene expression analysis	*In vitro / In vivo*	All Papers
Histological analysis	*In vivo*	*Paper I* and *III*
Trilineage assay	*In vitro*	*Paper II*

Micro CT analysis	*In vivo*	*Paper I* and III
Multipotency assay	*In vitro*	*Paper I* and III
PL preparation	*In vitro*	*Paper I*
Scaffold fabrication	*In vitro / In vivo*	*Paper III*
Subcutaneous implantation	*In vivo*	*Paper I* and III

4.5.1 Gene Expression Analysis via qPCR (ALL PAPERS)

Cell expression of relevant genes was analyzed at several stages throughout the entirety of the project. A summary of markers used for gene expression analysis via qPCR is shown in **table 4.**

Table 4. Overview of markers for gene expression analysis

	Full Name and Alias	Symbol	Species	Assay ID
Housekeeping genes (*All papers*)	Glyceraldehyde-3-phosphate dehydrogenase; ***GAPDH*** *(Paper I)*	GAPDH	Mouse	Mm 99999915_m1
	Glyceraldehyde-3-phosphate dehydrogenase; ***GAPDH*** *(Paper I)*	GAPDH	Human	Hs 02758991_g1
	Glyceraldehyde-3-phosphate dehydrogenase; ***GAPDH*** *(Paper II and III)*	GAPDH	Human	Hs 99999905_m1

Pluripotency genes (*Paper I and II*)	Homeobox NANO G; ***NANO G*** (*Paper I and II*)	NANO G	Human	Hs 02387400_g1
	Octamer-binding transcription factor 4; ***OCT-4; POU5F1*** (*Paper I and II*)	OCT4	Human	Hs 00999632_g1
	Sex determining region Y-box 2; ***SOX2*** (*Paper II*)	SOX2	Human	Hs 01053049_s1
Endoderm genes (*Paper II*)	GATA Binding Protein 4; ***GATA4***	GATA4	Human	Hs 00171403_m1
Mesoderm genes (*Paper II*)	Mesoderm Posterior BHLH Transcription Factor 1; ***MESP1***	MESP1	Human	Hs 00251489_m1
	Odd-Skipped Related Transcription Factor 1; ***OSR1***	OSR1	Human	Hs 01586544_m1
	HOP Homeobox; ***HOPX***	HOPX	Human	Hs 05028646_s1
Ectoderm genes (*Paper II*)	Paired Box 6; ***PAX6***	PAX6	Human	Hs 01088114_m1
	Retinal Homeobox Protein Rx; ***RAX***	RAX	Human	Hs 00429459_m1
Osteogenic Genes (*Paper I and III*)	Alkaline Phosphatase; ***ALP*** (*Paper I*)	ALPL	Human	Hs 01029144_m1
	Bone Gamma-Carboxyglutamate Protein;	BGLAP	Human	Hs 01587814_m1

	BGLAP; OCN (*Paper I and III*)			
	Bone Morphogenic Protein; ***BMP2*** (*Paper I and III*)	BMP2	Human	Hs 00154192_m1
	Bone Sialoprotein 2; ***BSP; IBSP*** *(Paper I)*	BSP	Human	Hs 00913377_m1
	Collagen Type 1 Alpha 1 Chain; ***COL1; COL1A1*** (*Paper I*)	COL1A1	Human	Hs 00164004_m1
	Collagen Type 1 Alpha 2 Chain; ***COL1; COL1A2*** (*Paper I and III*)	COL1A2	Human	Hs 00164099_m1
	Runt-Related Transcription Factor2; ***RUNX2*** (*Paper I and III*)	RUNX2	Human	Hs 00231692_m1
	Secreted Phosphoprotein 1; ***SPP1*** (*Paper I*)	SPP1	Human	Hs 00960942_m1
Adipogenic genes (*Paper I and III*)	CCAAT Enhancer Binding Protein Alpha; ***C/EBPA; CEBPA*** *(Paper I)*	CEBPA	Human	Hs 00269972_s1
	Lipoprotein Lipase; ***LPL*** (*Paper I and III*)	LPL	Human	Hs 00173425_m1
	Peroxisome Proliferator Activated Receptor Gamma; ***PPARG*** (*Paper I and III*)	PPARG	Human	Hs 01115513_m1
Inflammatory genes *(Paper I)*	Interleukin-1 Alpha; ***IL-1α***	IL-1α	Mouse	Mm 00439620_m1
	Interleukin-1 Beta; ***IL-1β***	IL-1	Mouse	Mm 00434228_m1
	Interleukin-6; ***IL-6***	IL-6	Mouse	Mm 00446190_m1

	Interleukin-8; *IL-8*	CXCL15	Mouse	Mm 00441263_m1
	Tumor Necrosis Factor Alpha; ***TNFα***	TNFα	Mouse	Mm 00443260_m1
	Interleukin-1 Alpha; ***IL-1α***	IL-1α	Human	Hs 00174092_m1
	Interleukin-1 Beta; ***IL-1β***	IL-1	Human	Hs 01555410_m1
	Interleukin-6; ***IL-6***	IL-6	Human	Hs 00174131_m1
	Interleukin-8; ***IL-8***	CXCL8	Human	Hs 00174103_m1
	Tumor Necrosis Factor Alpha; ***TNFα***	TNFα	Human	Hs 99999043_m1
Senescence genes *(Paper I)*	Cyclin Dependent Kinase Inhibitor 2A; ***CDKN2A***	CDKN2A	Human	Hs 00923894_m1
	Pleiotrophin; ***PTN***	PTN	Human	Hs 01085691_m1
	Poly(ADP-Ribose) Glycohydrolase; ***PARG***	PARG1	Human	Hs 00191351_m1
Mesenchymal stem cell genes *(Paper I)*	Thy-2 Cell Surface Antigen; ***THY1; CD90***	CD90	Human	Hs 00174816_m1

4.5.2 Surface Marker Expression via Flow Cytometric Analysis (ALL PAPERS)

Throughout this thesis, cells were characterized via flow cytometric analysis of specific surface antigens. The collected data was analyzed using a software for flow cytometry data analysis (FlowJo, LLC, USA). A summary of markers analyzed can be found in **table 5**.

Table 5. List of cell surface antigens analyzed via flow cytometry.

	Markers
Markers for MSC characterization *(paper I and III)*	CD14 CD34 CD44 CD45 CD73 CD90 CD105 HLA-A, HLA-B, HLA-C, HLA-DR
Markers for iPS characterization *(paper II)*	SOX2 OCT4

4.5.3 Multilineage Differentiation Potential

Osteogenic differentiation capacity (Paper I and III)

To induce osteogenic differentiation, cells were cultured in osteogenic medium for 21 days, and subsequently fixed and stained for extracellular mineral deposition via Alizarin Red staining solution (Sigma-Aldrich). Cells maintained in their respective culture medium served as controls.

Adipogenic differentiation capacity (Paper I and III)

To induced differentiation towards the adipogenic lineage, cells were cultured in adipogenic medium for 14 days. Cells were then either fixed and stained with Oil Red O staining solution (*paper I*), or assessed for expression of adipogenic related genes, *CEBPA* (*paper I*), *PPARG* and *LPL* (*paper III*) via qPCR. Cells maintained in their respective culture medium served as controls.

Chondrogenic differentiation capacity (Paper III)

To induce differentiation towards the chondrogenic lineage, cell pellets were maintained in chondrogenic medium (StemPro, Invitrogen) for 28 days, after which the pellets were embedded in Tissue-Tek compound (Tissue-Tek, O.C.T Compound, Sakura Finetek Europe B.V) and cryo-sectioned (MNT cryostat, MNT, SLEE medical GmbH, Olm, Germany). Sections were then stained with Alcian Blue (Sigma-Aldrich), and counter stained with Nuclear Fast Red (Sigma-Aldrich).

4.5.4 3D Osteogenic Potential of iMSC (Paper III)

To investigate *in vitro* osteogenic potential, iMSC were seeded on PLATMC scaffolds and osteogenically induced, after which multiple analyses were performed including ALP activity (normalized to the amount of DNA per corresponding sample), Alizarin Red staining of extracellular calcium deposition, and gene expression analysis of early and late osteogenic markers.

4.6 *IN VIVO* METHODS

4.6.1 Animal Model and Surgical Procedure

In *paper I*, NOD/SCID mice (17-21g) (Harlan Laboratories, Indianapolis, IN), between the ages of 6 and 8 weeks, were used to evaluate and compare the *in vivo* inflammatory responses and osteogenic potential of BM-MSC expanded in either PL supplemented medium or (AB + FGF) supplemented αMEM. Prior to surgical procedure, BCP were pre-wet overnight in medium (200 µl of PL or (AB+FGF) supplemented αMEM). The following day, BM-MSC, at passage 4, were detached and subsequently seeded onto the granules at a density of 7.5 x 10^3 cells per mg of BCP, after which cell/scaffold constructs were ready for implantation. BCP granules were implanted in subcutaneous pouches on the dorsum of the mice; either alone (controls) or in combination with BM-MSC. Mice were anaesthetized by inhalation of isoflurane (IsobaVet, NJ, USA) combined with O_2, after which the skin was disinfected with 70% ethanol. The back of the mice was then shaved and 2 skin incisions (approx. 1 cm each) were made in the midline along the vertebral column with sterile instruments. Following the incision, 3 – 4 pouches were made; 1 – 2 on each side of the midline, where cell/scaffolds were subsequently implanted. Samples were collected at two different time points; 1 and 11 weeks, at which the mice were euthanized by inhalation of an overdose of CO_2 gas, and samples were removed with the surrounding soft tissues (**Figure 7**), and immediately frozen in RNA later (Invitrogen, Thermo Fisher Scientific) at a temperature of -80°C. Half of each specimen was prepared for histological analysis, and the other half was processed for gene analysis. Each mouse in both the time points was implanted with the following: BCP granules without cells (**control group**), PL expanded BM-MSC seeded on BCP granules (BCP+BM-MSC+PL) (**PL group**), and AB-FGF expanded BM-MSC seeded on BCP granules (BCP+BM-MSC+AB+FGF) (**AB group**).

In *paper III*, NSG mice (The Jackson Laboratory, Bar Harbor, Maine) between the ages of 6 and 8 weeks, were used to evaluate the osteogenic potential of iMSC in both xenogenic and xeno-free conditions. Approximately 2 x 10^6 cells were seeded onto PLATMC scaffolds and cultured in osteogenic differentiation medium (described above) for 7 days prior to implantation. Cell/scaffold constructs (12 per group) were

implanted in subcutaneous pouches in the dorsum of the mice. Mice were anaesthetized by inhalation of isoflurane (IsobaVet) combined with O_2, after which the skin was disinfected with 70% ethanol. Two skin incisions (approx. 1 cm each) were made in the midline along the vertebral column with sterile instruments. Four pouches were then eventually made, 2 on either side of the midline, where cell/scaffold constructs were implanted. After 8 weeks, mice were euthanized via inhalation of an overdose of CO_2 gas, and samples collected along with surrounding soft tissue. Collected samples were stored in RNA later at a temperature of -80°C. Samples were cut in half, where one half was used for micro-CT and histological analysis, and the other for gene expression analysis. Previously isolated BM-MSC, cultured separately in FBS/PL supplemented osteogenic medium, served as controls [145].
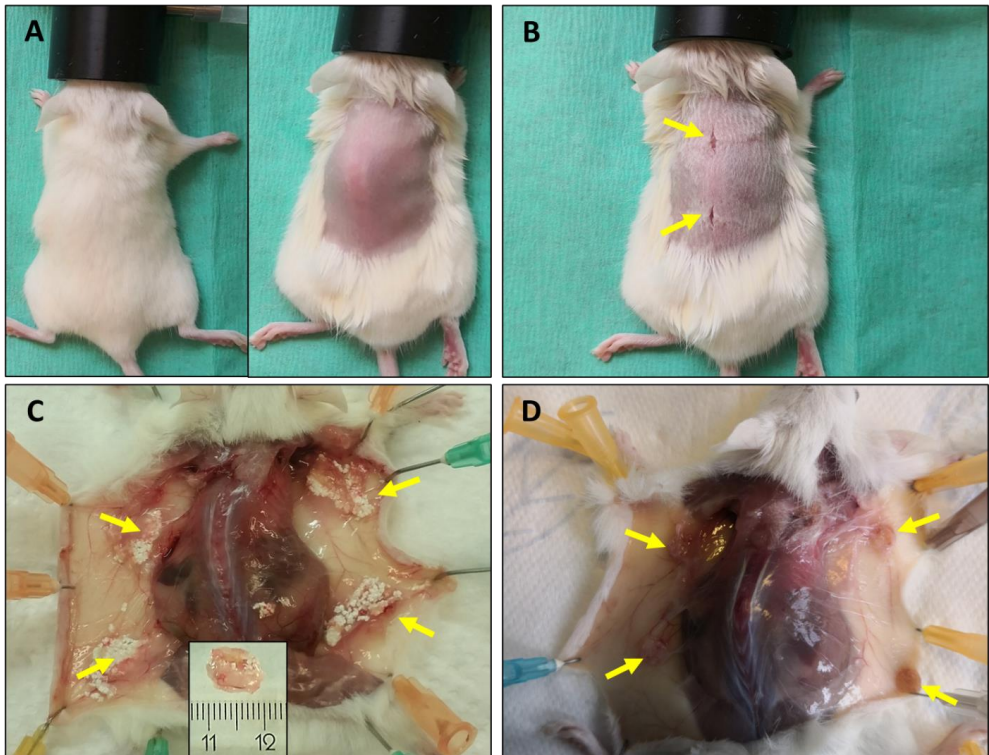
Figure 7. *In vivo* surgical procedure

A) Representative* images showing the *in vivo* mouse model following the administration of anesthesia (left) and removal of dorsal fur (right). **B)** Representative* image of the surgical incisions made (yellow arrows). **C)** *Paper I in vivo* samples (yellow arrows) prior to sample collection. **D)** *Paper III in vivo* samples prior to sample collection. *Images from *paper I* surgical procedure.

4.6.2 Micro-CT

In *paper III*, *in vivo* samples were collected and scanned via the SkyScan 1172 (Bruker microCT, Kontich, Belgium). Images were reconstructed via the NRecon reconstruction software (Bruker microCT), and 3D image analysis (CTAn, Bruker microCT) was performed to determine percent object volume.

4.6.3 Histology

In ***paper I*** and ***III***, *in vivo* samples were collected and fixed in 4% paraformaldehyde (Sigma-Aldrich) prior to being embedded in paraffin. Samples were then serially sectioned and stained with hematoxylin and eosin (H&E).

4.7   DATA PRESENTATION AND STATISTICAL ANALYSIS

qPCR gene expression data was analyzed using the $\Delta\Delta Ct$ method and presented as fold changes relative to a relevant control in the form of bar graphs and heatmaps. Surface marker expression via flow cytometry analyses were presented as histograms. Statistical analyses were performed via IBM SPSS Statistics (SPSS Inc.). Data are presented as mean values (+/- standard deviation). Statistical significance was determined via an independent samples T-test when comparing two groups, and one-way analysis of variance (ANOVA) when comparing more than two groups. A p-value of <0.05 was considered statistically significant.

5. RESULTS AND DISCUSSION

In this thesis we were able to develop a protocol devoid of xenogenic supplements for the reprogramming of donor-matched dermal and oral fibroblasts into iPS, and their eventual differentiation into iMSC. Although the xeno-free protocol lowered reprogramming efficiency, it ultimately supported the reprogramming procedure, and subsequently the stemness of the generated iPS (all xeno-free iPS showed typical pluripotent characteristics). The xeno-free protocol also supported the differentiation of iPS into iMSC; cells which satisfied the requirements set by the ISCT. Finally, the findings from both *in vivo* and *in vitro* analyses demonstrated that both PL and FBS effectively support the osteogenic differentiation of iMSC.

For the purpose of generating iPS, and their eventual differentiation to iMSC, we aimed to develop a xeno-free iPS/iMSC generation protocol. iPS were first discovered by Shinya Yamanaka in 2006, where he introduced the Yamanaka factors, OCT4, SOX2, KLF4 and c-Myc, into somatic cell nuclei via viral transduction [59, 60]. The original protocol is relatively flawed however, as c-Myc is now a known oncogene, and viral transduction is a known integrating protocol [41]. Hence, we improved on the Yamanaka protocol by using a different set of transcription factors, thereby replacing c-Myc. Furthermore, after a thorough search of the literature, we proceeded to utilize a non-integrating, episomal plasmid delivery method to introduce our specific selection of factors into cell nuclei [41]. Following the selection of an appropriate reprogramming method, we shifted our attention to cell culturing conditions. As previously mentioned, due to its xenogenic nature, FBS is a supplement to culture media which fails to meet the standard set by GMP guidelines [102]. GMP compliant alternatives to FBS include the xeno-free supplements HS and PL, both of which have previously demonstrated the ability to support the proliferative and differentiation capacity of MSC [19, 113, 129]. Hence, prior to developing and evaluating an iPS/iMSC generation protocol, it was vital to determine an optimal xeno-free supplement for cell culture. Therefore, we proceeded to evaluate two separate xeno-free culturing conditions: PL and HS.

5.1 Human AB Serum vs Human Platelet Lysate

While the effects of HS and PL are well documented, most studies compare them to FBS and rarely to each other. Previous reports have demonstrated that 10% HS is comparable to FBS in MSC isolation and expansion [113]. Similarly, media supplemented with PL has been shown to promote the expansion and proliferation of MSC more efficiently than when supplemented with FBS [126, 129]. As both protocols are relatively popular alternatives to FBS and are deemed to be at the very least comparable to FBS, we proceeded to evaluate the two xeno-free protocols. Therefore, in *paper I* the focus was on investigating the impact of two separate xeno-free isolation/culture conditions, namely PL and AB-FGF (referred to as *AB+FGFhigh* in *paper I*) supplemented conditions, on the stemness and osteogenic potential of BM-MSC. Despite the varying results, both culturing protocols effectively maintained the stemness and multipotency of the BM-MSC. Primarily, all cells expressed the pluripotency markers *OCT4* and *NANOG*, with expression being slightly higher in the cells expanded in PL as shown in *paper I*. This may suggest that PL maintains the cells in a more primitive nature as compared to AB-FGF. Similar findings have been observed previously, where MSC expanded in PL showed a higher expression of *OCT4* and *NANOG* in relation to MSC expanded in FBS [146, 147]. Our findings in *paper I* also revealed higher *IL6* expression in PL expanded BM-MSC relative to AB-FGF expanded BM-MSC [148]. *IL6* is an inflammatory cytokine, naturally expressed by MSC, known for its role in bone homeostasis, and is integral in maintaining MSC stemness, and therefore possibly affects gene expression of pluripotent markers. Previous reports have demonstrated the downregulation of *IL6* expression during MSC differentiation [149, 150]. This specific interleukin also enhances MSC proliferation, a process which is inversely correlated to differentiation [150, 151]. It is also suggested that *IL6* induces the activation of ERK1/2 cascade; a signaling pathway involved in cell proliferation and differentiation [150, 152]. Our *in vitro* results revealed higher osteogenic and adipogenic differentiation capacities when cells are expanded in AB-FGF when compared to PL. As proliferation always precedes differentiation, and FGF has been shown to increase proliferation rate, it's viable to assume that FGF likely had a major role in these results [153, 154]. Interestingly, however, Huang et al. also found

that, besides increasing proliferation, FGF also maintains the cells in a proliferative state and thereby delaying osteogenic differentiation. In their study however, they used a relatively high concentration of 300ng/ml of FGF as opposed to our 10ng/ml [154]. FGF has also been reported to maintain the multilineage differentiation capacity of MSC [155]. The results in our study might also be correlated with the relatively higher expression of *IL6* by BM-MSC in PL (as *IL6* maintains stemness and inhibits differentiation), which in turn, might be correlated with the increased expression of *OCT4* and *NANOG*. In line with these findings, previous studies report that *IL6* expression inhibits adipogenic and chondrogenic differentiation [150]. Despite the sense in assuming that an elevated *IL6* expression would naturally work against osteogenic differentiation, previous results surrounding the matter are inconclusive [150]. Altogether, these findings further support the notion that MSC stemness is maintained in PL culturing conditions.
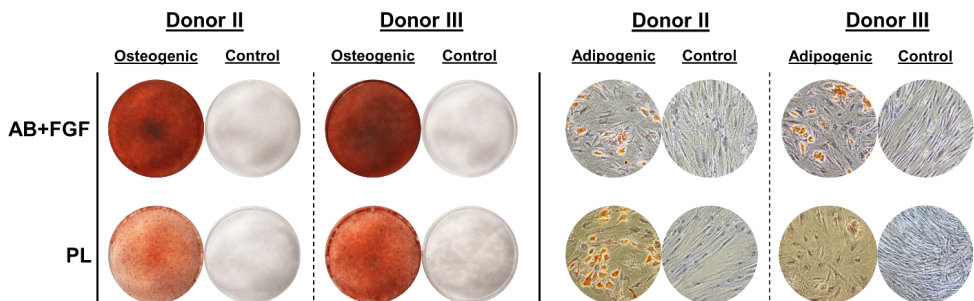
Figure 8. Osteogenic and adipogenic differentiation of BM-MSC

Alizarin Red and Oil Red O staining after induced differentiation of cells in both xeno-free conditions.

When investigating osteogenic potential, after 7 days *in vivo*, cells of the AB group demonstrated higher expression of early osteogenic markers: *RUNX2*, *COL1,* and *ALP*. This may suggest an earlier start to the osteogenic differentiation process by the cells of the AB group, which could be attributed to the effects of FGF. As FGF increases proliferation [153, 154], contact inhibition likely occurs faster leading to an earlier start to the differentiation process. Alternatively, the maintenance of stemness induced by PL might be resulting in delayed differentiation [149, 150]. Consequently, after 11

weeks, the majority of the AB group samples developed mature mineralized bone, while the PL group generally showed no mature bone formation (**Figure 9**). Instead, they revealed areas of 'permineralized' dense connective tissue around the margins of the BCP granules, with only a few samples developing areas of immature mineralized bone [156]. Perhaps a longer *in vivo* period for the PL group samples would have resulted in comparable bone formation in relation to the AB group. Prins et al. conducted a similar study, involving a total of 9 donors, but instead comparing BM-MSC expanded in PL to those expanded in FBS [157]. They found that their PL group resulted in ectopic bone formation in all donors (9 out of 9 donors; 40/49 scaffolds formed bone) as opposed to 6 out of 9 donors (38/49 scaffolds formed bone) in their FBS group. The three donors that demonstrated bone formation for only the PL cultured MSC, displayed a relatively low percentage of bone. Furthermore, inter-donor variation most certainly plays a role in these findings, as all three of the above-mentioned donors were female, and two of them were above the age of 60. Due to the smaller morphology observed and the more rapidly dividing cells, they speculate that cells cultured in PL are more immature than those in FBS and therefore have a greater differentiation ability. It is also important to mention that in their study, unlike our own, cells were osteogenically induced for 7 days before implantation, hence the relatively high overall bone formation data. Numerous other studies have also reported the ability of platelet derived products to enhance osteogenic differentiation of MSC in relation to FBS [19, 129, 133-135]. In this thesis, taken together with the *in vitro* findings, BM-MSC displayed osteogenic differentiation capabilities in both humanized conditions, albeit higher when cultured in AB-FGF. The delayed/lesser osteogenic differentiation ability in the PL group might be explained by a tendency of cells to retain a more non-differentiating phenotype.

AB+FGF   **PL**

A

B

C

	Week 1		Week 11	
	% of animals which formed bone	Average bone area (µm^2)	% of animals which formed bone	Average bone area (µm^2)
BCP alone	0 %	0	0 %	0
AB+FGF	0 %	0	83 %	1.40
PL	0 %	0	17 %	0.05
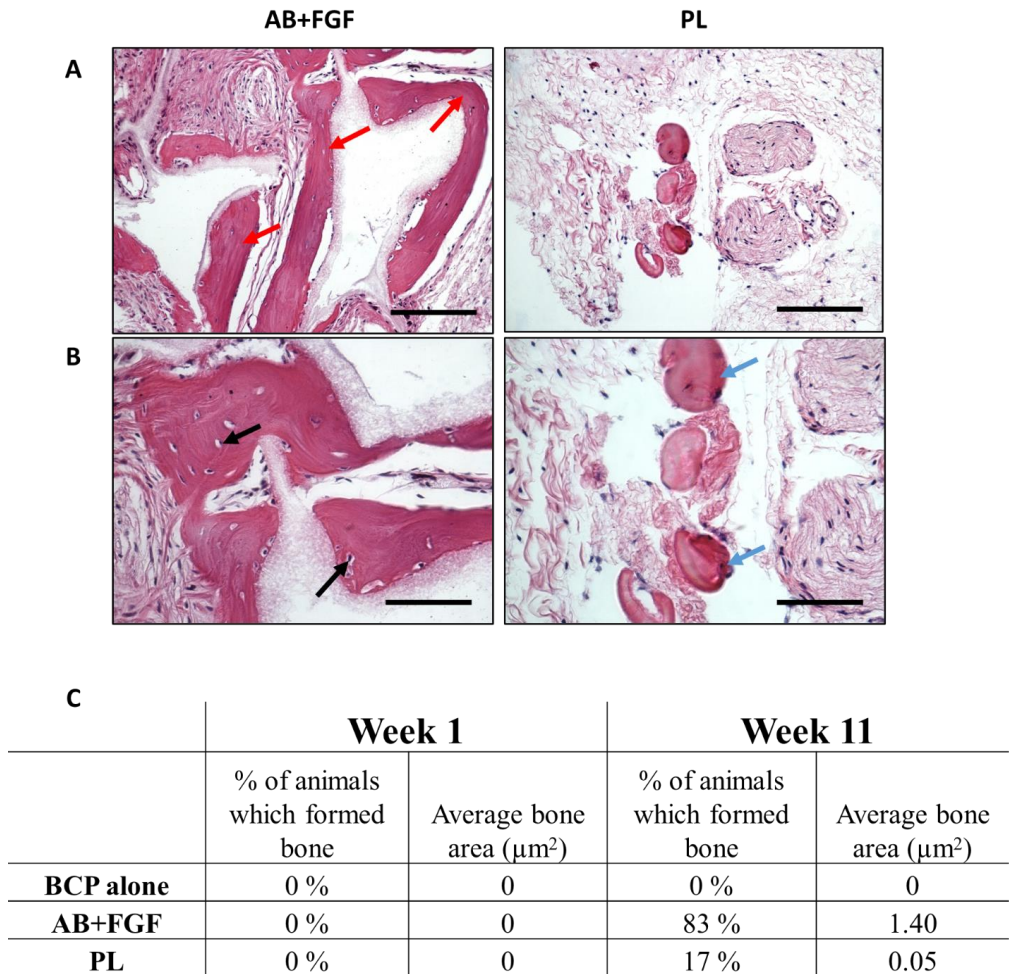
Figure 9. Bone formation *in vivo*

Representative histological images of cell/scaffold constructs after 11 weeks of subcutaneous implantation *in vivo*, showing mature mineralized bone (red arrows), osteocyte lacunae (black arrows) and immature bone like regions (blue arrows). **A)** Magnification x20; Scale bar = 100 µm. **B)** Magnification x40; Scale bar = 50 µm. **C)** Histomorphometrical analyses of the frequency and quantity of bone formed in the different implant groups.

Although the results of AB-FGF expanded BM-MSC are promising, it is important to highlight that FGF likely had a sizeable influence on the findings [118, 119]. This is

clearly evident in *paper I*, where different concentrations of FGF resulted in different experimental outcomes. While growth factors are generally essential to the regenerative process, exogenous growth factors present with multiple limitations [158, 159]. Major limitations of these factors include low protein stability, rapid clearance and short circulating half-life, rapid rate of cellular internalization. Protein integrity can also be compromised by fluctuations in temperature, pH *etc*., during long term storage [158]. Such variables aren't easily controlled or accounted for, making standardization difficult regardless of the initial concentration. Further challenges include difficulty in purification, and high cost of production [158].

The use of HS generally presents additional concerns. Primarily, a large amount of autologous blood is required for efficient long-term expansion of cells [160]. Regarding allogeneic HS, the lack of availability from blood banks has greatly hindered popularity and overall use [161]. Following the conclusion of this study, we decided to proceed with PL as the xeno-free supplement of choice in the remaining studies (*paper II* and *III*). As previously mentioned, a multitude of studies, from within our group and beyond, have demonstrated the ability of PL to support MSC expansion and osteogenic differentiation in both *in vitro* and *in vivo* settings [19, 129, 133-135]. Shanbhag et al. demonstrated superior osteogenic properties of BM-MSC and ASC, via Alizarin Red staining and ALP activity assays, when cultured in PL centered medium as opposed to FBS [129]. Prins et al. was able to showcase the ability of PL expanded BM-MSC to lead to the development of new bone subcutaneously at a higher rate than FBS expanded BM-MSC. Furthermore, our group has also demonstrated the clinical efficacy of using PL expanded BM-MSC for new bone formation [29]. Following *in vitro* expansion, BM-MSC combined with BCP granules were inserted subperiosteally onto a patients resorbed alveolar ridge. After 4 – 6 months of healing, significant bone formation was observed; sufficient for dental implant installation. Similarly, Gomez-Barrena et al. demonstrated new bone formation in 26/28 patients when 5% PL expanded BM-MSC combined with BCP were surgically delivered to non-union defects in the femur, tibia, and humerus [162]. Such findings demonstrate the safety and feasibility of utilizing PL for MSC expansion in both preclinical and clinical

settings. Besides the aforementioned drawbacks of HS, and benefits of utilizing PL, the decision to continue with PL as the xeno-free supplement of choice was based on a variety of other factors, including (i) practicality, as PL doesn't require the addition of exogenous growth factors, (ii) availability, as PL can be produced from expired pooled platelet concentrates, and (iii) future applicability [121, 122, 124].

5.2 Choice of media supplement influences reprogramming efficiency

In *paper II*, the focus was on generating iPS via a PL-centered, xeno-free protocol, and comparing them to iPS generated via a xenogenic protocol. Prior to evaluation, it is critical that iPS generation is conducted under standardized conditions. For instance, Hynes et al. previously conducted a study where iMSC from different iPS lines were characterized and evaluated [163]. However, the iPS used were generated in two separate laboratories, with separate sets of transcription factors. Furthermore, the iPS were generated from different individuals, *i.e.*, not donor matched, meaning there will most certainly be inherent differences between the cells. Such lack of standardization only adds to the many variables affecting such a complex procedure. On the other hand, A major strength in this paper is the standardized conditions that the cells were subjected to throughout the entirety of the study. All donor-matched fibroblasts were expanded in PL supplemented DMEM, and separately in FBS supplemented DMEM, simultaneously from the time of isolation up until approximately 7 days post transfection (**Figure 5**). Furthermore, the cells were all transfected in the same laboratory, with the same cocktail of transcription factors via the same method of delivery. This standardization allows for an efficient comparison of both iPS generating protocols.

Table 6. Data on the reprogramming of fibroblasts expanded in FBS/PL supplemented medium.

Fibroblasts	Success rate (Number of reprogramming procedures attempted)		Successful reprogramming		First day of iPS colony collection	
DONOR 1	**FBS**	**PL**	**FBS**	**PL**	**FBS**	**PL**
DERMAL	1 (100%)	3 (33%)	Yes	Yes	32	33
BUCCAL	1 (100%)	2 (50%)	Yes	Yes	34	35
GINGIVAL	1 (100%)	9 (11%)	Yes	Yes	32	39
DONOR 2	**FBS**	**PL**	**FBS**	**PL**	**FBS**	**PL**
DERMAL	2 (50%)	3 (33%)	Yes	Yes	28	31
BUCCAL	9 (11%)	9 (0%)	Yes	N/A	77	N/A
GINGIVAL	5 (20%)	4 (25%)	Yes	Yes	31	62

From the very beginning we faced difficulties when attempting to reprogram the different fibroblasts. Reprogramming efficiency within the different groups was generally quite low, with some groups not producing any iPS colonies, and others requiring several attempts before successfully producing iPS (**Table 6**). Such findings are to be expected considering the extremely low reprogramming efficiencies generally reported in the literature [88]. Schlaeger et al. conducted a study comparing several different reprogramming methods in reprogramming efficiency (defined in their study as the number of emerging colonies per starting cell number), success rate (percentage of samples for which a minimum of 3 iPS colonies emerged) etc [66]. Episomal plasmid transfection was found to have a 93% success rate (lentiviral -100% and Senda-viral – 94%). However, this particular method also scored the lowest reprogramming efficiency at 0.013%. While offering the advantage of being integration free, the issue of low reprogramming efficiency when using episomal plasmids remains a major drawback. Chow et al. encountered similar difficulties when attempting to reprogram dermal fibroblasts obtained from adult dogs using a CytoTune iPS Reprogramming kit (Sendai-viral kit from Life Technologies). Ultimately, a mere two colonies were produced, and only one was deemed viable for further expansion

[164]. Currently, most iPS related studies fail to report such data, which highlight the first major obstacle in iPS generation [41, 88]. Our data clearly indicates that a xeno-free protocol is less supportive of the reprogramming process. Previous reports also seem to support this notion. For instance, Sung et al. found that PL was less favorable for inducing cellular reprogramming in human amniotic fluid stem cells, and reprogramming efficiency was significantly higher in the cells cultured in medium supplemented with FBS [139]. The exact reason for this decreased efficiency is unclear, however, similar to the findings in *paper I*, we speculate that it is somehow attributed to the innate ability of PL to maintain the nature of cells, and thus, somewhat preventing any form of cellular change, *i.e.*, differentiation and reprogramming [146, 147].

5.3 Dermal fibroblasts more susceptible to reprogramming than oral fibroblasts

Dermal fibroblasts were first used to generate human iPS and are currently the most common cell type used in reprogramming procedures [60]. Multiple groups have since generated iPS from various different cell types [72, 77, 79, 80]. Overall, choosing an optimal cell type for iPS generation depends on a variety of factors including reprogramming efficiency, invasiveness of collection procedure, difficulty in cell isolation, cost effectiveness etc. Reprogramming data gathered in *paper II* indicates that DF are generally more susceptible to reprogramming than both BF and GF. Such inherent disparities in susceptibility have been demonstrated in previous studies and must be addressed when considering the optimal cell type for iPS production. For example, keratinocytes are reportedly more easily reprogrammed than fibroblasts, and similarly, dental pulp stem cells yield more iPS colonies than BM-MSC [72, 75]. Yan et al. also reported difficulties when attempting to reprogram GF via various transduction protocols, including lentiviral and retroviral transduction, with none of the protocols yielding any iPS colonies [75]. As is the case with a large portion of iPS studies, details regarding the number of reprogramming attempts made are missing. Perhaps continued attempts would have ultimately led to the successful generation of iPS from GF. The specific reasons as to why we see this higher susceptibility in DF as

compared to oral fibroblasts is unclear, however inherent differences between the fibroblasts is expected to play a role [165, 166]. Inter-donor differences, such as age etc., may also lead to variations in reprogramming susceptibility and the subsequent iPS [167-170]. For instance, cells obtained from the elderly are associated with an increased risk of iPS abnormalities and a decrease in reprogramming efficiency, likely related to the upregulation of *p21*[171-173]. A similar upregulation has been seen in relatively late passage fibroblasts, indicating a negative correlation between cell passage/culture and reprogramming efficiency [173]. The relationship between cell proliferation and differentiation also seems to affect iPS generation, as previous reports suggest that successful reprogramming is easier to accomplish in actively dividing cells in relation to non-dividing cells [174]. This theory, however, has not been confirmed, as contradictory findings indicate that a decrease in cell proliferation is beneficial to induction of pluripotency [175]. The phenomenon of inter-donor disparities is evident in our data, where the BF and GF samples from D1 were considerably more susceptible to reprogramming than those from D2. As the sample size in this study is too small to adequately address donor variability on iPS generation, future studies must be performed to determine the correlation.

5.4 Induction of pluripotency is feasible in both xenogenic and xeno-free protocols

In this project, cell reprogramming was deemed a success when reprogrammed cells developed morphological features typical for iPS, in the form of colonies surrounded by a reflective border (**Figure 10A-F**). Following successful induction of pluripotency, no major differences were seen between the geno/phenotype of the iPS-PL and iPS-FBS. All iPS groups morphologically formed colonies (stable colonies) comparable to that of ESC as shown previously [168, 176]. The iPS also expressed the pluripotency genes *OCT4*, *SOX2* and *NANOG*; known to play a key role in inducing/maintaining iPS stemness, with expression in general being slightly lower in iPS-PL [177]. This lower expression, however, did not translate at protein level, where expression of OCT4 and SOX2 was comparable in both groups. The iPS also displayed the ability to differentiate towards the three primary germ layers, by expressing endoderm,

mesoderm, and ectoderm lineage specific genes (see **Fig. 6** in *paper II*). The effect of cell source on iPS geno/phenotype showed only minor differences between the groups with no particular observable trend. Hence, these minor variations could not be directly attributable to variations in the initial cell source. The findings above coincide with previous results highlighting the phenotypical and genotypical similarities of iPS when obtained from different sources and/or via different protocols [72, 74, 163]. Overall, these results confirm the ability to generate pluripotent cells with ESC like characteristics via both xenogenic and xeno-free protocols.
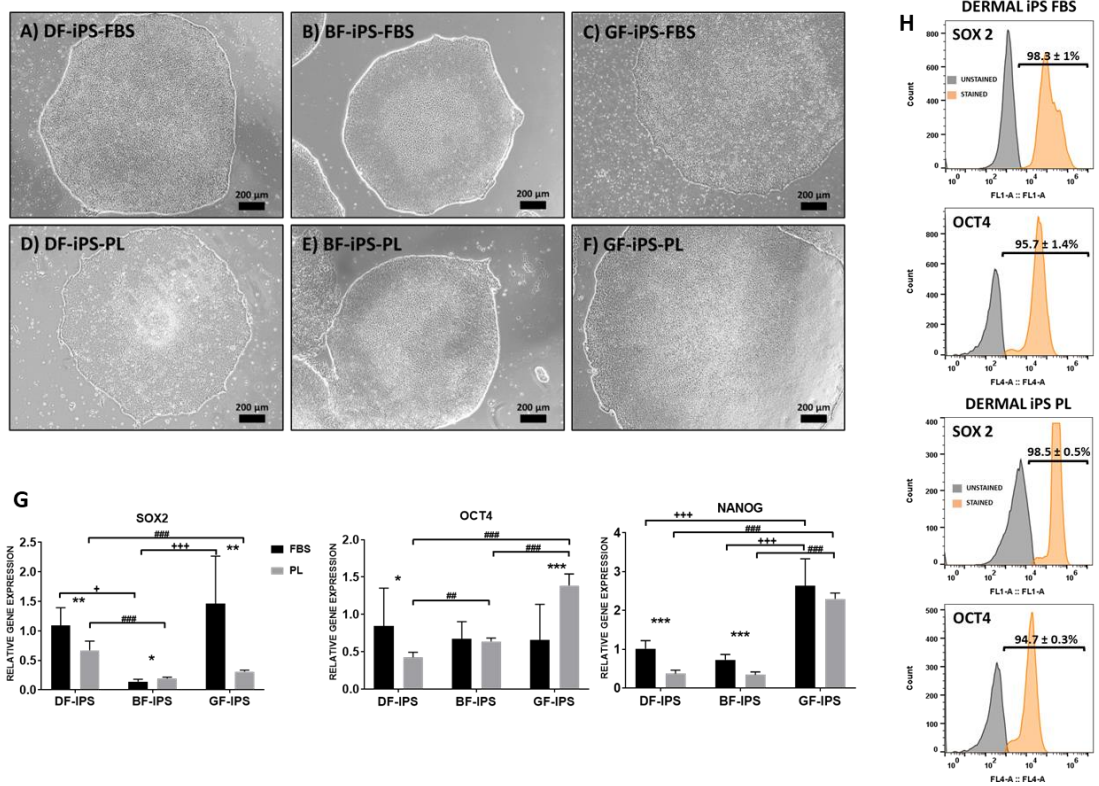

Figure 10. Characteristics of generated iPS

(A-F) Light microscopy images demonstrating iPS morphology generated via FBS and PL culturing protocols. **G)** Relative gene expression of SOX2, OCT4, and NANOG by the different iPS. **H)** Flow cytometric analysis revealing expression of intracellular markers SOX2 and OCT4. Figure showing only analysis of dermal iPS, as a general

representation of expression patterns of all the iPS groups. For full flow cytometric figure, see **Fig. 5** in *paper II*.

One major area of concern surrounding pluripotent cells is the presence of aberrations within the cell genome. In the case of iPS, the acquisition of these genomic alterations may occur as a result of the transfection procedure, long-term culture of cells, or merely being passed down from parent somatic cells [68, 178, 179]. Hence, it is important to ensure that no alterations are caused by the introduction of new supplementation into iPS generation protocols. When analyzing the genomic state of human pluripotent cells, the literature points to chromosomes 1, 12, 17, 20 and X as being generally the most affected [68, 69]. In our study however, a different set of chromosomes were more commonly affected within the iPS, namely chromosomes 1, 5, 11 and 13 (**Figure 11**). The CNVs were relatively low, except in the cases of both DF-iPS-PL and DF-iPS-FBS, where duplications of several segments were identified throughout chromosome 1. In most cases, genetic variations in iPS have been reported to be caused by somatic mosaicism in the culture of their parent fibroblasts [68], and in some instances 50% of CNVs found in iPS were also found in the parental fibroblasts [68, 180]. This however is not the case with the DF-iPS, as genetic analysis revealed no such duplications in chromosome 1 of their parent fibroblasts (see **Additional file** in *paper II*). Therefore, it seems that this aberration in particular is caused by either the reprogramming process, or alternatively, by the long-term culture/maintenance of the iPS [179]. During cell reprogramming, multiple colonies generally develop within the well containing transfected fibroblasts, and in many cases each colony is collected and cultured separately (**Figure 6C**). Hence, since long-term culture of iPS can cause aberrations, one may speculate that different clones of the same cell line, *i.e.*, different iPS colonies from the same source, may have a different chromosomal profile. Ultimately, despite the links between chromosome 1 aberrations and tumor formation [181], the majority of the evidence points to the harmlessness of such abnormalities in iPS. Such aberrations are common, as both ESC and iPS are often genetically unstable. Furthermore, teratomas can develop from both genomically normal and abnormal iPS,

and there is little evidence linking genomic abnormalities with tumor formation [68, 69].
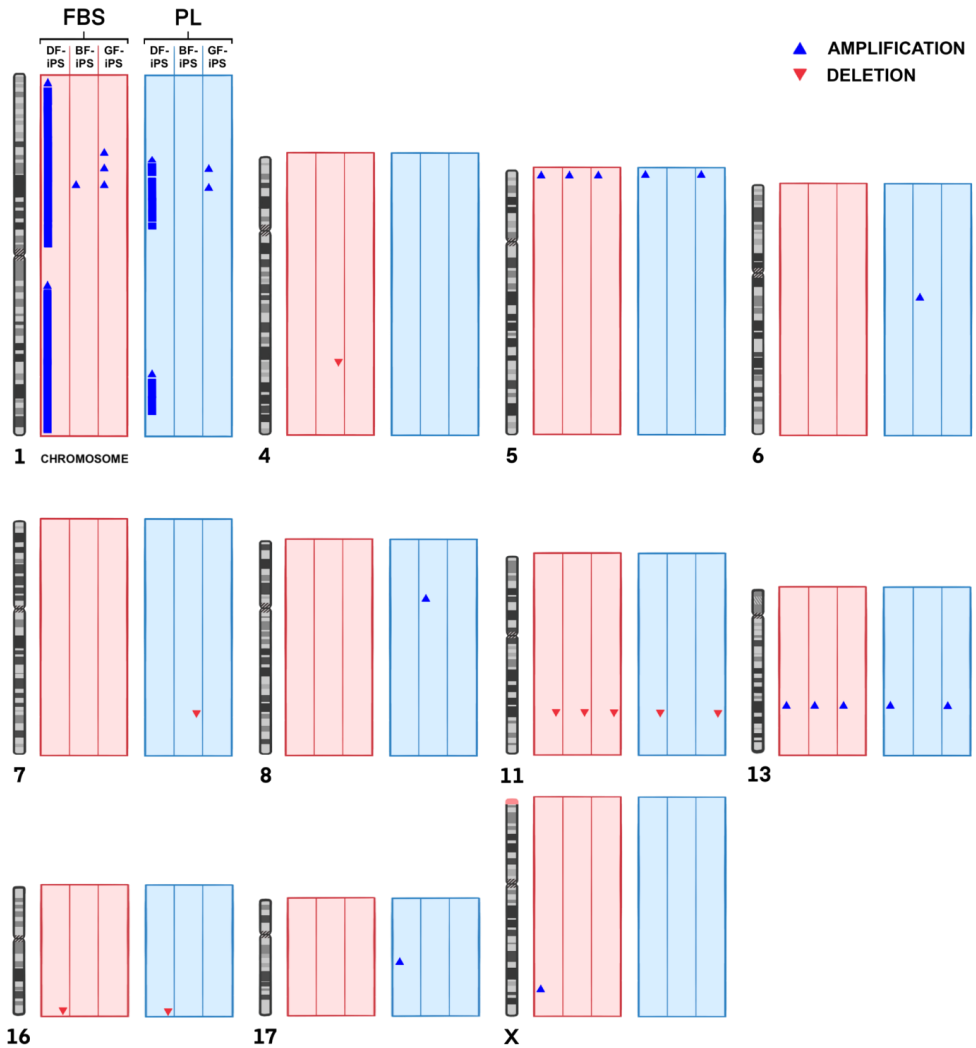
Figure 11. Chromosomal stability of iPS

Representative* figure displaying chromosomal CNVs of the different iPS. Arrows and bars represent the gain (blue) or loss (red) of a chromosomal region. *Illustration only depicts chromosomes affected with CNVs; for full set of chromosomes, see *paper II*, *Fig. 7*. This figure was created using Procreate 5.2 on iOS software.

5.5 Xeno-free iMSC show typical MSC characteristics

In *paper III* we differentiated the iPS obtained in *paper II*, into iMSC using a simple differentiation protocol. As has been previously reported [97-100], both sets of generated iMSC (xeno and xeno-free) developed a phenotype in accordance with the criteria set by the ISCT [45]. Furthermore, they displayed considerably lower expression of pluripotent genes relative to their parent iPS, further cementing their non-pluripotent, MSC status (**Fig. 2** in *paper III*). Our findings showed iMSC displaying a spindle shaped fibroblast morphology. The iMSC, in both PL and FBS, also demonstrated a typical MSC immunophenotype of >90% expression of the surface markers CD73, CD90, and CD105, and >90% negative expression of the markers CD34, CD45 and HLA-DR (**Figure 12** and **Fig. 3** in *paper III*).
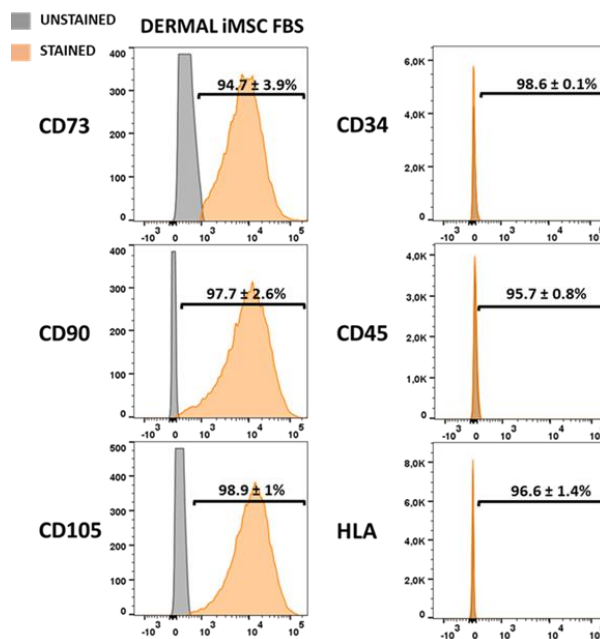
Figure 12. Surface marker expression of iMSC

Flow cytometric analysis* of iMSC revealing surface marker expression of MSC markers CD73, CD90 and CD105, and lack of expression of hematopoietic markers HLA-DR, CD34 and CD45. *Figure showing only analysis of dermal iMSC-FBS, as a

general representation of iMSC expression patterns in this study. For full flow cytometric analysis, see **Fig. 3** in *paper III*.

As a defining feature of MSC status, multipotency was confirmed by several analyses highlighting the differentiation capabilities of the iMSC (**Fig. 4** in *paper III*). Although all displayed multipotent capacity, the iMSC-PL appeared to demonstrate a higher ability for adipogenic and chondrogenic differentiation. Their higher tendency towards adipogenic differentiation was determined by the increased expression of known adipogenic markers PPARG, heavily expressed by adipocytes during adipogenesis, and LPL, predominantly expressed by brown adipocytes [182, 183]. The higher tendency towards a chondrogenic lineage was determined via formation of what appeared to be more compact pellets and an apparent increase in GAG formation (molecules involved in physiological functions of cartilage) [184]. On the other hand, their osteogenic differentiation ability was clearly inferior to the iMSC-FBS, as is evident by the degree of Alizarin Red staining. In this case, it seems that PL, relative to FBS, pushes the cells towards a chondrogenic and adipogenic lineage. Studies involving BM-MSC and adipose derived MSC, however, have reported a higher osteogenic capacity when cultured in PL than when cultured in FBS [126, 129, 133]. Such discrepancies may be attributable to the heterogeneity in PL batches [19], or perhaps it's simply cells of different nature reacting differently to various supplements in culturing protocols [145, 185].

Overall, within each group, only minor differences were observed in differentiation capacity between the different iMSC. Such variation is supported by previous reports describing the capabilities of MSC derived from different populations. BM-MSC have been shown to have a higher capacity for osteogenic and chondrogenic differentiation than adipose-derived MSC, which in turn showed a higher capacity for adipogenic differentiation [129, 145]. These findings are to be expected considering MSC possess a tendency to differentiate towards a lineage related to their original microenvironment, *i.e.*, adipose derived-MSC towards an adipogenic lineage, BM-MSC towards an osteogenic lineage etc [186]. Along with the role of the microenvironment, it is suggested that MSC epigenetics also aid in controlling cell differentiation and

proliferation [186, 187]. Although at this stage of the project, all iMSC analyses were done at an *in vitro* level, the effects of the microenvironment and epigenetics of cells prior to isolation/differentiation must be taken into consideration. In relevant cases, donor variation plays a major role as well [167]. Ultimately, these findings demonstrate the ability to generate iMSC from different iPS via both xenogenic and xeno-free protocols.

5.6 Osteogenic potential of iMSC

5.6.1 *In Vitro* 3D Model

BM-MSC reside naturally in bone, a 3D substrate, where they respond to specific stimuli which affects their behavior, e.g., FGF growth factor secreted by osteoblasts, [188]. Therefore, in attempts to better mimic *in vivo* conditions, we analyzed the osteogenic behavior of our generated iMSC in a 3D environment. Furthermore, due to the inherent osteogenic nature of bone marrow [189], we compared iMSC to BM-MSC (controls) which were also propagated under similar xenogenic and xeno-free conditions. Their osteogenic potential was evaluated via alizarin red staining, ALP activity and gene expression analysis of specific osteogenic markers. ALP is an enzyme that plays an integral role in the early stages of osteogenesis by increasing inorganic phosphate and thereby facilitating mineralization [190, 191]. Additionally, ALP decreases the concentration of extracellular pyrophosphate, an inhibitor of mineral formation [190]. The observed increase in mineral deposition (similar to 2D staining results) and ALP activity in the iMSC-FBS, relative to the iMSC-PL, may point to an earlier start to the osteogenic differentiation process (See **Fig. 5** and **6** in *paper III*). A similar trend was observed in the BM-MSC, as xenogenic BM-MSC also showed higher degrees of staining and ALP activity relative to their xeno-free counterparts. The gap, however, was considerably less than was observed between the xeno and xeno-free iMSC, *i.e.*, BM-MSC-PL showed significantly higher degrees of alizarin red staining and ALP activity compared to iMSC-PL. Our *in vitro* findings seemingly point to a delayed osteogenic differentiation process induced by PL culturing conditions on iMSC, which is not equally reflected on BM-MSC. On the other hand, the BM-MSC

and iMSC in FBS showed comparable results despite significantly higher ALP activity by the BM-MSC (**Fig. 6** in *paper III*). These results are supported by previous studies highlighting comparable osteogenic capabilities of iMSC and BM-MSC when propagated in FBS conditions [192, 193]. These studies also report that BM-MSC possess superior multilineage potential than iMSC due to their much greater capacity for adipogenic/chondrogenic differentiation [192, 193].

To further support our findings, we measured the expression of early and late osteogenic markers, after 7 and 21 days of induction (**Fig. 7** in *paper III*). The early markers included *RUNX2* and *COL1*, which are osteoprogenitor markers expressed during the early phase of osteogenic differentiation [194, 195]. *RUNX2* also induces the commitment of MSC towards the osteogenic lineage [196, 197]. The late markers consisted of *BMP2* and *BGLAP*, also known as osteocalcin, which are expressed relatively late in the differentiation cycle and are more related to osteoblastic activity [198, 199]. In general, both BM-MSC-PL and iMSC-PL showed higher gene expression of osteogenic markers at the 21 day mark compared to their xenogenic counterparts. This may indicate an increase in osteogenic potential at gene level. This apparent increase, however, fails to translate at protein level as is evident by the alizarin red staining and ALP activity findings. Alternatively, the observed expression patterns may point to a relatively earlier start to the differentiation process induced by FBS culturing conditions. For example, within the FBS group, expression of *RUNX2*, *COL1* and *BMP2* was either comparable or lower at the 21 day time point relative to the 7 day time point. This may indicate that peak expression of the aforementioned markers was most likely prior to day 21. In the PL group, *RUNX2* and *COL1* expression was upregulated after 21 days. Nevertheless, expression may still be on its way down. This theory is supported by previous 2D results from previous studies where BM-MSC-FBS were found to reach peak expression levels of RUNX2 and COL1 at approximately 14 days [145, 193]. These results also might explain the results of the Alizarin Red staining/ALP activity. Perhaps the culture of these cells in FBS conditions speeds up the osteogenic differentiation process or alternatively, as previously observed via the lower reprogramming/differentiation capacities in *paper I* and *II*, it might be that PL culture merely slows it down by maintaining the stemness of cells. This might also

suggest that continued differentiation of the cells in PL would eventually yield similar, and perhaps even superior results. These findings highlight the importance of using adjunctive methods to gene expression analyses. Primarily, gene expression does not necessarily translate at protein level, *i.e.*, not entirely useful for predicting future protein expression [200]. Furthermore, when measured at a single time point, gene expression analysis fails to provide a full picture, including crucial information regarding expression patterns etc., e.g., whether expression was higher or lower prior to this singular time point. Therefore, such analyses may be better utilized as a supplement to protein analyses, and thereby bridging the gaps. Analyzing the effect of the initial cell source on osteogenic potential revealed only minor differences. However, no particular pattern was detected, and thus, none of the variations could be directly attributable to differences in tissue of origin of the parent iPS.

5.6.2 *In Vivo* Model

In vivo microenvironments comprise many factors which function in tandem to directly, or indirectly, influence cell behavior. These factors include, but are not limited to, neighboring cells, extracellular matrix, cytokines, hormones as well as mechanical forces [187]. When it concerns MSC, these factors regulate cell proliferation and differentiation, and therefore different microenvironments have different effects on MSC function. In turn, a plethora of factors are also released from MSC which influence the surrounding environment [187]. Hence, in attempts to adequately evaluate the *in vivo* osteogenic capacity of iMSC, we implanted cell/PLATMC constructs subcutaneously in the dorsum of immunodeficient mice, with BM-MSC serving as controls. In general, relatively common host responses to foreign bodies are characterized by chronic inflammatory infiltration along with the formation of a fibrous capsule. In the early stages, the inflammatory process includes the recruitment of neutrophils and macrophages, which also tend to be present in the later stages [201]. As NSG mice are extremely immunodeficient, lacking B and T cell function as well as NK function, a reduced inflammatory response to implanted foreign cells is to be expected [202, 203]. Nevertheless, the implanted iMSC/scaffold constructs in this study set off a similar host response to that of the implanted BM-MSC/scaffold

constructs. H&E staining revealed a response consisting of an inflammatory infiltrate and a fibrous capsule surrounding partially degraded scaffold material (PLATMC). With respect to osteogenic potential, ectopic environments, by nature, are not ideal, as they lack endogenous bone forming cells and osteogenesis related signals [204]. However, when coupled with a bio-inert scaffold, ectopic models eliminate most of the osteogenic related variables, providing an ideal setting for assessing the osteogenic induction/formation capacity of implanted cells [17]. Following sample collection after 2 months *in vivo*, gene expression analysis revealed the expression of osteogenic markers by all groups (**Fig. 9** in *paper III*). Similar to the *in vitro* findings (21-day time point), osteogenic gene expression was generally higher in the PL-samples relative to FBS-samples, potentially indicating a greater osteogenic capacity. On the other hand, as observed in *paper I* and *III* (see *in vitro* gene expression data and discussion), gene expression data may once again indicate a delayed differentiation response by cells when cultured in PL. BM-MSC also generally demonstrated higher osteogenic marker expression within each group; an expected finding considering the osteogenic nature of BM-MSC [189]. Despite the expression of osteogenic genes, bone formation analysis generally revealed the absence of mineralized tissue in all groups (**Figure 13**). Histological sections revealed the formation of a mixture of loose and dense connective tissue fibers, areas which might be considered 'pre-mineralized' [156], but ultimately negligible mineralization was observed (also confirmed by micro-CT analysis, see **Fig. 10** in *paper III*). Overall, since no mineralization was observed in any of the samples, these results indicate that osteogenic capacity doesn't necessarily translate from an *in vitro* to an *in vivo* setting.
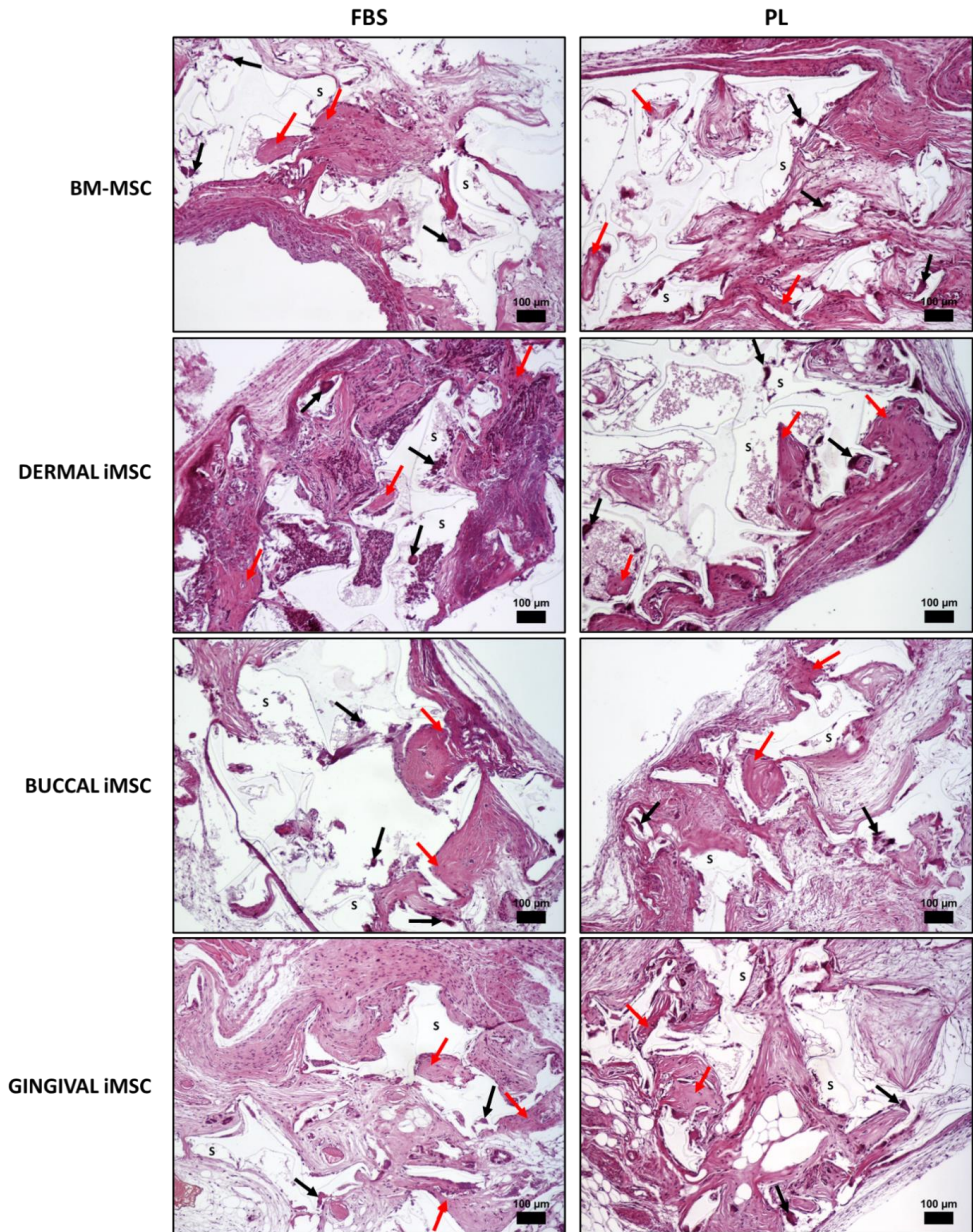
Figure 13. Histological images of *in vivo* mineralization

Histological images of implanted samples after 8 weeks. Images revealing areas of relatively dense connective tissue matrix (red arrows) and scaffold material (S) surrounded by multinucleated giant cells (black arrows). Scale bar = 100 µm.

Despite their osteogenic nature, BM-MSC were also incapable of forming bone ectopically, unlike what was observed in previous findings, including those in *paper I* [205]. The reason for these differing results is likely related to the scaffold of choice, and perhaps, the animal model selected. In *paper I*, similar to many other studies, a calcium phosphate-based ceramic was used as a carrier as opposed to a bio-inert scaffold [205]. These types of scaffolds are generally selected due to their chemical similarity with bone and their osteoinductive properties [206-208]. While providing an advantage in terms of bone forming capacity, the activity of bioactive scaffolds makes it difficult to solely assess the osteogenic ability of cells. Moreover, the *in vivo* model of choice likely factors in on the differing outcomes seen in *paper I* and *III*. Both NOD/SCID and NSG mice are immunodeficient strains characterized by the absence of functional B and T cells [202, 203]. Due to a lack of IL-2-receptor-γ chain, NSG mice are also deficient in NK cell function, making them considerably more immunodeficient than NOD/SCID strains, greatly diminishing their inflammatory response to foreign cells [202]. Since the initial phase of bone regeneration is an inflammatory phase, lack of an immune response certainly hinders subsequent osteogenic outcomes. In addition to the influence of scaffold material, this phenomenon might explain why the *in vivo* BM-MSC samples in *paper III* failed to form bone, as opposed to those in *paper I.* In this experimental setting, in the absence of an osteoinductive scaffold, osteogenically differentiated xenogenic and xeno-free iMSC/BM-MSC were incapable of inducing mineralization.

6. CONCLUSIONS

Based on the findings of this thesis, the conclusions derived from each study are as follows:

1. Both AB and PL are viable xeno-free alternatives to FBS for MSC expansion. For bone regeneration purposes, both expansion protocols support the osteogenic differentiation of BM-MSC.

2. Both FBS and PL culturing protocols support the generation of iPS from matched dermal, buccal, and gingival fibroblasts. Relative to FBS, the use of PL in culture medium decreases reprogramming efficiency. Oral fibroblasts were generally more difficult to reprogram than dermal fibroblasts. Once successfully generated, cell source and expansion conditions have little effect on iPS geno/phenotype.

3. iMSC can be successfully generated from iPS, via both FBS and PL differentiation protocols. All generated iMSC satisfied requirements for 'MSC' status, as set by the ISCT. According to the data gathered via *in vitro* and *in vivo* analyses, a PL culturing protocol supports the osteogenic differentiation of iMSC. Tissue of origin has little bearing on iMSC geno/phenotype, and osteogenic potential in both *in vitro* and *in vivo* settings.

7.   FUTURE PERSPECTIVES

The findings in *paper II* highlight a major obstacle, in the form of low reprogramming efficiency, generally encountered when attempting to reprogram somatic cells. If iPS are to be used in the future, it is vital to better understand and address the reasons behind this low reprogramming efficiency. Furthermore, it would be relevant to assess and compare the reprogramming efficiency of different non-integrating reprogramming methods when implemented on donor-matched sources.

In this thesis, the use of PL as a supplement to basal culture media lowers reprogramming efficiency when compared to FBS. We speculate that this is attributed to the innate ability of PL to maintain the nature of the cells and as a result, resisiting the changes necessary for successful reprogramming to occur. This must be assessed further in a larger sample size to confirm and determine the reasons/mechanism as to why PL cultured fibroblasts reprogram less efficiently than FBS cultured fibroblasts. Moreover, the question of whether this phenomenon is exclusive to fibroblasts should be assessed, *i.e.*, do other cell types show similar reprogramming efficiencies when cultured in PL as opposed to FBS. Hence, other relatively non-invasive and easily isolated cells should be considered for inclusion in future assessments, e.g., urine-derived stem cells, hair follicle keratinocytes etc. This would also present the opportunity to determine a more optimal cell type for xeno-free iPS generation.

In *paper III*, the ectopic *in vivo* experiment yielded relatively no mineralization. Since this particular experiment was designed to assess the osteogenic potential of the cells alone, parameters that would have been more favorable for mineralization were purposefully excluded. In the future, it would be of interest to incorporate a setting more conducive to bone formation, followed by a similar assessment to that performed in *paper III*. For instance, a calvaria defect model instead of an ectopic subcutaneous one. BCP scaffolds, such as those used in *paper I*, could also be used in place of the bio-inert scaffolds. Overall, further evaluation is warranted to build on the findings presented and discussed in this thesis.

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9. Original Papers

Paper I

Impact of humanized isolation and culture conditions on stemness and osteogenic potential of bone marrow derived mesenchymal stromal cells.

Salwa Suliman, **Hassan R.W. Ali**, Tommy A. Karlsen, Jerome Amiaud, Samih Mohamed-Ahmed, Pierre Layrolle, Daniela E. Costea, Jan E. Brinchmann, Kamal Mustafa.

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OPEN

Impact of humanised isolation and culture conditions on stemness and osteogenic potential of bone marrow derived mesenchymal stromal cells

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Therapeutic potential of human bone marrow stromal/stem cells (hBMSC) must be developed using well defined xenogenic-free conditions. hBMSC were isolated from healthy donors (n = 3) using different isolation and expansion methods. Donor I was isolated and expanded by either bone marrow directly seeded and cells expanded in 10% AB human serum (AB) + 5 ng/ml fibroblast growth factor-2 (FGF2) [Direct(AB + FGF$_{low}$)] or Ammonium-Chloride-Potassium Lysing Buffer was used before the cells were expanded in 10% AB + 5 ng/ml FGF-2 [ACK(AB + FGF$_{low}$)] or Lymphoprep density gradient medium was used before the cells were expanded in 10% AB + 5 ng/ml FGF2 [Lympho(AB + FGF$_{low}$)] or bone marrow directly seeded and cells expanded in 10% pooled platelet lysate plasma (PL) + heparin (2 I/U/mL) [Direct(PL)]. Groups for donors II and III were: Direct(AB + FGF$_{low}$) or 10% AB + 10 ng/ml FGF2 [Direct(AB + FGF$_{high}$)] or Direct(PL). HBMSCs were assessed for viability, multi-potency, osteogenic, inflammatory response and replicative senescence in vitro after 1 and 3 weeks. Pre-selected culture conditions, Direct(AB + FGF$_{high}$) or Direct(PL), were seeded on biphasic calcium phosphate granules and subcutaneously implanted in NOD/SCID mice. After 1 and 11 weeks, explants were analysed for inflammatory and osteogenic response at gene level and histologically. To identify implanted human cells, in situ hybridisation was performed. hBMSC from all conditions showed in vitro multi-lineage potency. hBMSCs expanded in PL expressed stemness markers in vitro at significantly higher levels. Generally, cells expanded in AB + FGF2 conditions expressed higher osteogenic markers after 1 week both in vitro and in vivo. After 11 weeks in vivo, Direct(AB + FGF$_{high}$) formed mature ectopic bone, compared to immature mineralised tissues formed by Direct(PL) implants. Mouse responses showed a significant upregulation of IL-1α and IL-1β expression in Direct(PL). After 1 week, human cells were observed in both groups and after 11 weeks in Direct(AB + FGF$_{high}$) only. To conclude, results showed a significant effect of the isolation methods and demonstrated a relatively consistent pattern of efficacy from all donors. A tendency of hBMSC expanded in PL to retain a more stem-like phenotype elucidates their delayed differentiation and different inflammatory expressions.

Mesenchymal stromal/stem cells are progressively being used in all arenas of tissue engineering and cell-based therapies[1,2]. With regard to bone regeneration, human bone-marrow derived mesenchymal stromal/stem cells

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(hBMSC) present advantages over other sources of MSC[3] and over pluripotent cell types such as induced pluripotent stem cells due to their autologous mode of use, which require less extensive *in vitro* manipulation or ethical clearance, associated with a lower risk[4]. hBMSC are rare cells, population ranges from 0.001% to 0.01% of the total number of nucleated cells present in bone marrow[5]. Pertaining to this drawback, *in vitro* cell expansion in monolayers is the most commonly used approach to produce sufficient cell numbers prior to pre-clinical or clinical implantations. Despite the increasing number of clinical trials, culturing conditions for hBMSC are still under development[6]. There is substantial evidence that the *in vitro* expansion phase affects their phenotype, with considerable implications for the development of effective therapies. With hBMSC-based therapies overtaking clinical applications in bone regeneration and establishing a new clinical paradigm[1,2], the development of production methods in accordance with current Good Manufacturing Practices (GMP) is mandatory for a safe and efficient regeneration[6,7]. In compliance with the European Commission regulation 1394/2007, hBMSC are considered advanced therapy medicinal products in Europe[8].

Clinical translation trials in accordance with GMP require the use of a well-defined culture medium when expanding hBMSC to avoid adverse reactions in patients[6]. Foetal bovine serum (FBS) is derived from the whole blood of bovine foetuses and it is a rich source of essential growth factors. These include platelet derived growth factor (PDGF), transforming growth factor beta 1 (TGFβ-1), fibroblast growth factor 2 (FGF2), vascular endothelial growth factor (VEGF), insulin-like growth factor (IGF), growth hormones and albumin, making it the optimum and most broadly used supplement for expansion of hBMSC[9]. However, it comes with safety concerns such as zoonotic infections since it contains enogeneic antigens as well as ethical concerns[9,10]. In addition, the concentrations of growth factors in FBS are difficult to control between production batches, and even clinical-grade FBS is reported to show variability between its inherent composite of bioactive factors[9]. To address these issues, alternative animal-free strategies are currently being developed for the provision of nutrients and attachment factors for culture and expansion of hBMSC. These are generally divided into chemically defined media, and 'humanised' supplements derived from human blood derivatives. The proposed derivatives include: autologous or allogeneic human serum, human platelet derivatives, cord blood serum and human plasma derivatives[11].

When comparing hBMSC expanded using human serum to those cultured using FBS, promoted proliferation and enhanced gene expressions with genomic stability were portrayed[12]. Studies mainly using autologous serum revealed potential for expansion and osteogenic differentiation of hBMSC; however this potency was shown to be age dependant[13]. Reports on allogeneic serum have been contradictory, and pooling of blood samples seems to reduce variability[12,14]. Use of autologous serum presents with limitations, for instance availability of large quantities required for clinical applications[15]. Therefore, alternatives such as pooled human serum from type AB donors were introduced.

The physiological role of blood platelets in tissue repair justifies the use of their derivatives in regeneration. Human platelet lysate (PL) can be obtained from platelets using different procedures (*e.g.* thrombin activation, freeze/thaw platelet lysis or sonication). It contains an extensive variety of growth factors and cytokines such as VEGF, FGF2, epidermal growth factor (EGF), IGF, PDGF and TGF-β1 in higher levels relative to human serum[16]. PL has shown superior efficacy when compared to FBS-propagated cultures. hBMSC were shown to be more responsive to chondrogenic and adipogenic stimulation when cultured in PL compared to FBS[17]. Among the advantages of PL is the constancy of its bioactivity even when using expired platelet concentrates[18], proving comparable proliferation and osteogenic potential when used to culture hBMSC[19]. PL is highly dependent on the preparation method, donor age and blood profile, which makes it difficult to accurately characterise its constituents; however, pooling can reduce variances[20]. To avoid the fibrin clots from forming when human PL is added to the culture medium, which contains calcium, heparin is usually added to human PL. The levels of heparin have shown to variably interfere with the growth rate of cells[18]. Furthermore, concerns of immunological responses and transmission of human infections are also valid as for other human-derived media supplements.

Variation in expansion conditions influences the efficacy and differentiation potential of hBMSCs. This is an important consideration when designing tissue engineering and regenerative studies. Most reports comparing the human alternatives to FBS have provided contradictory results in their proliferation and differentiation potencies. Only few reports have done an inter-comparison among the different human alternatives to highlight the actual differences in efficacy[21]. Increasing cell yield, while maintaining stemness, represents a significant challenge for the *in vitro* expansion of clinical grade hBMSC. Recently, we reported a Phase 1 clinical trial to regenerate dentoalveolar bone defects where autologous hBMSC were expanded in GMP-grade PL from human pooled platelet concentrates as growth factor supplement[22]. In attempts to improve these protocols and transfer technologies, the current study compares different isolation methods of hBMSC and further expansion in different human-derived culture media, namely, human AB serum (AB) supplemented with FGF2 or PL. To evaluate the regenerative therapeutic capacity of these cells expanded using different isolation and culture conditions, a systematic assessment was carried out both *in vitro* and *in vivo* in an ectopic rodent model.

Methods

Pooled human platelet lysate preparation. PL plasma was prepared according to published protocols[23] with minor modifications. Briefly, pooled platelets from 4 donors suspended in platelet additive solution was spun at 1700 g at room temperature (RT). The resulting pellet was re-suspended in 10 mL Octaplas AB plasma (Octapharma AS, Jessheim, Norway) and frozen at −20 °C. This constituted one unit. After thawing, platelets from 19 units were pooled and adjusted to a final volume of 4.8 L with Octaplas AB, and subjected to a second freeze-thaw-cycle before being centrifuged at 4000 g at 4 °C for 15 min to remove platelet cell wall fragments, and subsequently frozen in aliquots. When PL was added to culture medium, 2 I/U per mL heparin was added, following previously optimised protocols[20], to avoid coagulation of the medium through clumping of the fibrinogen in the plasma. The platelets used to make PL are all originally donated for use for patients. However, if they are not used after 7 days of storage they are deemed unfit for use in patients, and will normally be destroyed, or used for

DONOR I	
Abbreviation	**Description**
Direct(AB + FGF$_{low}$)	Bone marrow was directly seeded onto flasks with α-MEM +10% AB serum +1% P/S and 5 ng/mL FGF2 (all from R&D)
ACK(AB + FGF$_{low}$)	Prepared with Ammonium-Chloride-Potassium (ACK) lysing buffer (Gibco) and cells were seeded with α-MEM +10% AB serum +1% P/S and 5 ng/mL FGF-2
Lympho(AB + FGF$_{low}$)	Bone marrow was centrifuged in a density gradient medium Lymphoprep (Stemcell Technologies) to isolate mononuclear cells before culturing in α-MEM +10% AB serum +1% P/S and 5 ng/mL FGF2 (all from R&D)
Direct(PL)	Bone marrow was directly seeded onto flasks with α-MEM +1% P/S (Gibco) +10% PL + heparin (2I/U per mL)

Table 1. Description of the different isolation and expansion conditions for Donor I. Abbreviations: α-MEM, alpha minimum essential medium; AB serum, human serum from type AB donors; P/S, penicillin/streptomycin, FGF2, fibroblast growth factor 2.

DONORS II and III	
Abbreviation	**Description**
Direct(AB + FGF$_{low}$)	Bone marrow was directly seeded onto flasks with α-MEM +10% AB serum +1% P/S and 5 ng/mL FGF2
Direct(AB + FGF$_{high}$)	Bone marrow was directly seeded onto flasks with α-MEM +10% AB serum +1% P/S and 10 ng/mL FGF2
Direct(PL)	Bone marrow was directly seeded onto flasks with α-MEM +10% AB serum +1% P/S +10% PL + heparin (2I/U per mL)

Table 2. Description of the different isolation and expansion conditions for Donor I and III. Abbreviations: α-MEM, alpha minimum essential medium; AB serum, human serum from type AB donors; P/S, penicillin/streptomycin, FGF2, basic fibroblast growth factor 2.

research. We have tested, and found that they may still be used to make PL, as the alternative is that these platelets are destroyed. The donors are anonymous to the research laboratory, and the platelet or platelet lysate is not being subject to any type of analysis. Ethical approval to use PL in culture conditions was obtained from the Regional Committees for Medical and Health Research Ethics in Norway (REK approval no. 2016/1266). All procedures were performed in accordance with the relevant guidelines and regulations in the blood bank.

Human bone marrow stromal/stem cells isolation and expansion. HBMSC were isolated from the bone marrow of three different healthy donors. A total of 30 to 60 mL of bone marrow was obtained by aspirations from the iliac crest under local anaesthesia, after ethical approvals were obtained from the Regional Committees for Medical and Health Research Ethics in Norway (REK approval no. S-07043a). All donors gave a written, informed consent and all procedures were performed in accordance with the relevant guidelines and regulations. The donors were 28, 37 and 37 years of age. The cells were characterised and assessed after isolation using different bone marrow processing conditions and expansion culture conditions. Briefly, the whole bone marrow from the first donor was divided equally and the different isolation and expansion conditions used are summarised in Table 1.

All methods involving humans were performed in accordance with the relevant guidelines and regulations.

The bone marrow from the 2nd and 3rd donors was equally divided and directly seeded without prior processing and expansion conditions are described in Table 2.

The cells were expanded for 2 passages at a laboratory at Norwegian Center for Stem Cell Research in Oslo University Hospital before they were transferred to the laboratory at University of Bergen for further *in vitro* differentiation and *in vivo* osteogenic potential evaluation. The viability of the cells before and after shipment was assessed using trypan blue dye.

Multipotent capacity *in vitro*. Cells were seeded ($3-5 \times 10^3$ per cm^2) in 12-well plates (NUNC, Thermo Fisher Scientific, Waltham, USA). Differentiation was induced 2–5 days from seeding (depending on the cells' confluency) using adipogenic medium for 2 weeks and osteogenic media for 3 weeks (all from StemPro, Thermo Fischer Scientific). For detection of adipogenic differentiation, cells were stained with Oil Red O stain, while mineralisation was detected by Alizarin red staining as described previously[24].

Multipotency, inflammatory response, osteogenic and adipogenic differentiation potential and replicative senescence *in vitro* at gene level. Using a Tissue RNA isolation kit (Maxwell, Promega, Madison, WI, USA), total RNA was isolated from *in vitro* osteogenically differentiated hBMSC cultures after 1 and 3 weeks and undifferentiated controls at 1 week only. Quantity and purity were checked using a Nanodrop spectrophotometer (Thermo Fisher Scientific). Total RNA was reverse transcribed according to manufacturer's instructions using the High-capacity complementary DNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA, USA). Quantitative real time PCR was conducted on a StepOne Plus system (Applied Biosystems),

using TaqMan gene expression assays (Applied Biosystems). Gene expression assays (Taqman) (Applied Biosystems) were used to detect mRNA levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), octamer-binding transcription factor 4 (Oct-4), NANOG and Cluster of Differentiation 90 (CD90) and the inflammatory markers interleukin 1 alpha (IL-1α), beta (IL-1β), interleukin 6 (IL-6) and 8 (IL-8) in the undifferentiated controls. mRNA levels of runt-related transcription factor 2 (RUNX2), collagen type 1 alpha 1 (Col 1α1), alkaline phosphatase (ALP), bone morphogenetic protein-2 (BMP-2), bone sialoprotein (BSP), osteocalcin (OC) and GAPDH were detected from osteogenically differentiated cultures at 1 and 3 weeks. mRNA levels of adipogenic markers, peroxisome proliferator-activated receptor gamma (PPARG), CCAT/enhancer-binding protein alpha (CEBPA) and lipoprotein lipase (LPL) were detected from adipogenically differentiated cultures at 2 weeks. To evaluate replicative senescence, mRNA level of upregulated genes during cellular aging, Rho GTPase activating protein 29 (PARG1/ARHGAP2) and cyclin dependent kinase inhibitor 2 A (CDKN2A) and downregulated gene pleiotrophin (PTN) were evaluated from osteogenically differentiated cells at 1 and 3 weeks. The data were analysed with a comparative C_T method and GAPDH served as an endogenous control. Primer details are summarised in Supplementary Table 1.

Surface markers evaluation. The results obtained from the *in vitro* characterisation determined the direct seeding isolation method for further evaluation of osteogenic efficacy *in vivo*. Before seeding on scaffolds, the cells from Direct(AB + FGF$_{high}$) or Direct(PL) were stained with mouse anti-human CD44-FITC (Bio SB), CD90-PE (eBioscience), HLA-A,B,C-APC (BD Biosciences, USA), HLA-DR-APC (eBioscience), CD105-FITC (Sigma), CD14-FITC (Sigma), CD73-PE (BD Biosciences), CD34-FITC (BD Biosciences), CD45-FITC (eBioscience) for 15 min at RT, before being washed and re-suspended in PBS. Mouse anti-human immunoglobulin isotype antibodies (eBioscience) were used as controls. Acquisition was performed using a BD LSRFortessa cell analyser (BD Biosciences) and data were analysed using FlowJo (V10, Flowjo LLC, Ashland, OR, USA).

Cell/scaffold constructs' preparation and subcutaneous implantation. HBMSC from Direct(AB + FGF$_{high}$) or Direct(PL) without being osteogenically differentiated were seeded on biphasic calcium phosphate (BCP) granules (MBCP; Biomatlante, France) ranging in size from 1–2 mm. Cells (7.5×10^3) per mg were seeded on 65 mg of BCP placed in a 96-well plate. Cell-free BCP (BCP alone) served as controls. The plates were then shaken in a plate shaker to allow distribution of the cells within the granules and incubated overnight at 37 °C and 5% CO_2. Two, 1 cm incisions, were made on the back of 8–10 weeks old non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice. The two groups of cell/scaffold constructs or the BCP alone groups were randomly distributed and each animal got a total of four subcutaneous implants (n = 8 for each group). The mice were sacrificed after 1 and 11 weeks. All animal experiments were approved by the Norwegian Animal Research Authority and conducted in strict accordance with the European Convention for the Protection of Vertebrates used for Scientific Purposes (FOTS no. 7894).

Osteogenic differentiation potential and inflammatory response *in vivo* at gene level. Total RNA was isolated from *in vivo* scaffolds 1 week post-implantation and PCR was performed as described previously for the *in vitro* samples. Taqman gene expression assays (Applied Biosystems, USA) were used to detect mRNA levels of GAPDH, RUNX2, Col1α2, ALP, IL-1α, IL-1β, IL-6 and IL-8 for human and mice. The data were analysed with a comparative CT method, with GAPDH as an endogenous control and BCP alone as the reference group.

Histological analysis and histomorphometry. After fixation in 4% paraformaldehyde, the harvested *in vivo* scaffold constructs were decalcified in EDTA and cut into half before being processed for paraffin embedding. Sections (3–4 μm) were stained with haematoxylin and eosin (H&E). The histological sections were scanned using an Aperio Scanscope Scanner (Aperio Vista, CA, USA) and viewed through Aperio ImageScope software program. Qualitative and semi-quantitative histological evaluations were carried out to assess the amount of bone formed. The frequency of bone formation was estimated as the number of scaffolds with newly formed bone related to the total number of implanted scaffolds.

***In-situ* hybridisation of the human Alu-sequence.** To identify the hBMSC in the cell/scaffold constructs harvested after the *in vivo* implantation, *in situ* hybridisation using the human-specific repetitive Alu sequence was performed as previously described[25]. Briefly, paraffin sections were treated for heat induced epitope retrieval for 20 h at 60 °C in citrate buffer 10 mM (pH 6) plus Tween 20 0.05% followed by 0.25% acetic acid in 0.1 M triethanolamine (pH 8) for 20 minutes at RT. Prehybridisation was performed for 3 hours at 56 °C in a hybridisation buffer containing 4 × Saline-Sodium Citrate buffer (SSC) (Sigma-Aldrich), 50% deionised formamide, 1 × Denhardt's solution, 5% dextran sulfate, 100 μg/ml salmon sperm DNA and molecular-grade water. Hybridisation buffer was refreshed with the addition of 70 nM custom DIG-labelled locked nucleic acid Alu probe 5DigN/5′-TCTCGATCTCCTGACCTCATGA-3′/3DigN (Exiqon, Vedbaek, Denmark) and then target DNA and the probe were denatured for 1 h at 70 °C. Hybridisation was carried out overnight at 56 °C. The hybridised probe was detected by immunohistochemistry using biotin-SP conjugated IgG fraction monoclonal mouse anti-digoxin (Jackson Immunoresearch, West Grove, Pennsylvania, USA) diluted 1/400 in Tris-buffered saline with Tween, 2% bovine serum albumin for 1 h at RT. Stretoperoxidase was added (1/400 in Tris-buffered saline with Tween) for 35 min at RT. All bound reactions were visualised with 3,3′ diaminobenzidine substrate (Dako, Les Ulis, Ile-de-France, France). Sections were counterstained with Gill-2 hematoxylin (Thermo Shandon Ltd, Runcorn, UK).

	Condition	Cell viability before shipment	Cell viability 24 h later
Donor I	Direct(AB + FGF$_{low}$)	97%	85%
	ACK(AB + FGF$_{low}$)	98%	91%
	Lympho(AB + FGF$_{low}$)	98%	91%
	Direct(PL)	92%	84%
Donor II	Direct(AB + FGF$_{low}$)	96%	94%
	Direct(AB + FGF$_{high}$)	96%	93%
	Direct(PL)	97%	92%
Donor III	Direct(AB + FGF$_{low}$)	98%	96%
	Direct(AB + FGF$_{high}$)	98%	95%
	Direct(PL)	98%	92%

Table 3. Viability of cells after shipment.

Statistical analyses. All data are presented as the mean values + standard error of the mean. Average values were analysed using SPSS Statistics 25.0 (IBM, Armonk, NY, USA). Data were tested for variance homogeneity and normal distribution and One-way ANOVA were followed by a multiple- comparison LSD test. Differences between the means were considered statistically significant when $p < 0.05$.

Ethics approval. Bone marrow samples were collected after ethical approvals were obtained from the Regional Committees for Medical and Health Research Ethics in Norway (REK Approval No. S-07043a). The samples were collected following an informed consent of the patients. All animal experiments were approved by the Norwegian Animal Research Authority and conducted in strict accordance with the European Convention for the Protection of Vertebrates used for Scientific Purposes (FOTS No. 7894).

Results

Cells' viability was maintained after 24 h shipment. The expanded hBMSC were transported within 24 hours from Oslo (Norway) to Bergen (Norway) at RT via DHL Express overnight courier. The majority of the cells cultured in different conditions expressed viability above 90%. Their viability on arrival at Bergen is summarised in Table 3.

hBMSC preserved variable degrees of stemness when cultured under different conditions.
Undifferentiated hBMSC expressed significantly higher levels of CD90 mRNA when they were isolated directly from bone marrow and expanded in PL for all donors. The transcription factors regulating pluripotency, NANOG and Oct-4, were significantly upregulated in the cells that were isolated directly from bone marrow and expanded in PL for all donors, followed by Lympho(AB + FGF$_{low}$) in donor I and Direct(AB + FGF$_{high}$) in donor II and III (Fig. 1a). Cells from all donors expanded under all different conditions tested showed multipotency when induced. The osteogenic differentiation was confirmed by presence of a mineralised matrix when stained for Alizarin red. Cells expanded with AB + FGF$_{high}$ were observed to have the strongest Alizarin red stain, as assessed macroscopically (Fig. 1b). The adipogenic lineage was defined by the formation of lipid vacuoles stained by Oil red (Fig. 1c).

Different isolation and culture conditions influenced the inflammatory responses *in vitro*. The mRNA of pro- and anti-inflammatory markers displayed variable expression among different groups. IL-1α, the pro-inflammatory marker showed the highest expression in Direct(AB + FGF$_{low}$) for all donors (Fig. 2). The lowest expression in donor I was detected in cells from Lympho(AB + FGF$_{low}$) (Fig. 2a). However, when only the Direct seeding method was compared for donor II and III, the lowest expression was seen in cells from Direct(PL) (Fig. 2b). The lowest expression for IL-1β was presented by cells from Direct(PL) for all donors.

IL-6 showed the highest significant expression in cells from Direct(PL); however for donor I and III the cells from Direct(AB + FGF$_{low}$) showed the highest expression, followed by cells from Direct(PL). The mRNA expression of IL-8 showed more consistency among the donors where cells expanded under AB + FGF portrayed the highest expressions.

Different isolation and culture conditions influenced the osteogenic differentiation potential of the cells *in vitro*. The mRNA expression of most of the osteogenic markers showed generally comparable trends among all isolation and cell culture conditions tested here (Fig. 3). The early transcription factor RUNX2 continued to be upregulated significantly from 1 to 3 weeks in all groups. After 1 week, cells isolated by direct method and cultured in AB + FGF showed the highest expression of RUNX2 for all donors. Early osteogenic marker, Col1α1, displayed a significant upregulation from 1 to 3 weeks in all groups except Direct(AB + FGF$_{high}$) for donor II which showed an inversed trend. Generally, cells grown in AB serum, regardless of the isolation method showed higher expression at both time points compared to cells in PL.

ALP mRNA expression was significantly downregulated for all groups after 3 weeks except for Direct(PL), where it was upregulated in cells from donors II and III. The highest expression at both time points was in cells from Lympho(AB + FGF$_{low}$) for donor I followed by cells from Direct(PL). For donor II and III, cells from Direct(PL) showed the highest significant expression at both time points.
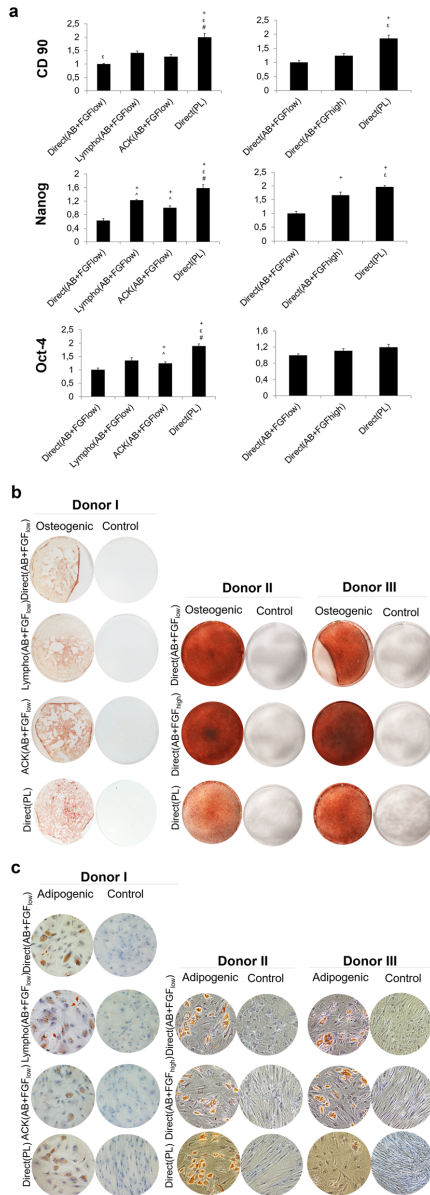
Figure 1. Multipotency characterisation *in vitro*. (**a**) Relative mRNA expression of MSC marker, CD90 and transcription factors regulating pluripotency, NANOG and Oct-4 after 1 week of non-induced culture after transport. Expression is presented relative to the Direct(AB + FGF$_{low}$) group (p < 0.05). (**b**) Alizarin stain showing cells from all culture conditions and from all donors differentiated consistently into the osteogenic lineage when induced. (**c**) Oil red O stain confirming lipid vacuoles when adipogenic lineage was induced.

The mRNA expression of BMP-2 showed a consistent significant upregulation after 3 weeks for donor I and donor III in all culture condition groups. However, a downregulation was observed after 3 weeks in donor II for most groups. The highest BMP-2 mRNA expression at both time points were seen from cells expanded in AB + FGF either by the ACK or Direct seeding method.
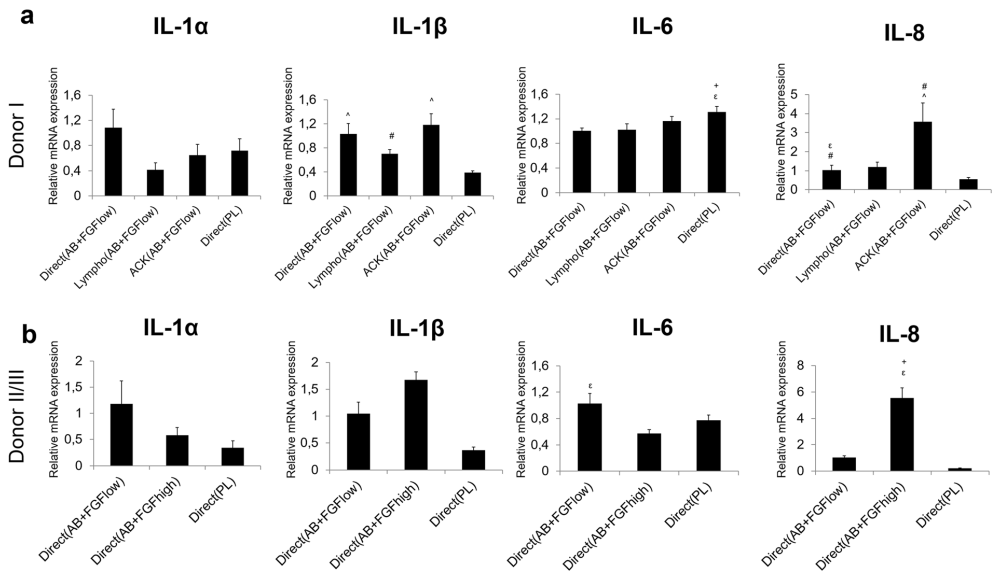
Figure 2. mRNA expressions of inflammatory markers *in vitro* 1 week post transport. Different groups evaluated from **(a)** donor I and **(b)** donor II/III. Y axes represent relative mRNA expressions for IL-1α, IL-1β, IL-6 and IL-8. Expression is presented relative to the Direct (AB + FGF$_{low}$) group, (p < 0.05).

BSP mRNA presented comparable trends of expression among all groups for all donors. There was a significant upregulation after 3 weeks in all groups, with the highest expression from cells grown in AB + FGF in general and specifically ACK(AB + FGF$_{low}$) for donor I. Osteocalcin, a late osteogenic marker, was significantly upregulated after 3 weeks along all culture condition groups for all donors. The highest osteocalcin mRNA expressions at both time points were seen from cells isolated and expanded in ACK(AB + FGF$_{low}$) for donor I and similary cells grown in AB + FGF from donor II and III. Direct(PL) cells showed the lowest expression of BSP and osteocalcin at both time points for all donors.

Different isolation and culture conditions influenced the adipogenic differentiation potential of the cells *in vitro*. The mRNA expression of the adipogenic markers was evaluated at 2 weeks and it showed largely comparable trends among all isolation and cell culture conditions tested, proving the adipogenic differentiation and thus multipotency of the cultured hBMSC (Fig. 4).

The highest expressions in all adipogenic markers (PPARG. CEBPA and LPL) were from Lympho(AB + FGF$_{low}$) cells from donor I and the lowest expressions from Direct(PL) cells. Cells grown in AB + FGF in general from donor III expressed higher levels compared to Direct(PL) in all markers. Donor II showed variable expressions among the three markers between the groups.

Different isolation and culture conditions influenced the replicative senescence of the cells. The mRNA expression of a combination of the commonly upregulated and downregulated genes associated with cellular aging were evaluated after 1 and 3 weeks of osteogenically differentiated cells cultured in the different conditions. Significant differences were observed between the different expansion conditions and between time points (Fig. 5). The mRNA expression of PTN at 1 week was comparable between all groups, however after 3 weeks there was an upregulation in all groups and significantly higher upregulation was observed in the AB + FGF groups in general in donor II and III. These results might elucidate the survival of Direct(AB + FGF$_{high}$) *in vivo* up to 11 weeks compared to cells cultured in Direct(PL). PARG1 was significantly expressed lower in Direct(PL) in donor II and III in week 1 and CDKNA2 showed similarly the lowest expression significantly in Direct(PL) in all donors at week 1. After 3 weeks, Direct(PL) showed the highest expression of PARG1 in donor II and III and AB + FGF groups generally showed higher expressions in CDKNA2 (Fig. 5).

Different culture conditions influenced the inflammatory and host response potential of the cells *in vivo*. Before *in vivo* implantation, cells from Direct(AB + FGF$_{high}$) and Direct(PL) showed comparable expressions of all the tested surface cell markers (data not shown) except for HLA-DR + where the percentage and intensity reduced considerably in cells from Direct(PL) for both donors II and III (Fig. 6a). Implanted hBMSC did not express any human inflammatory genes *in vivo* (data not shown). Mouse inflammatory responses showed an upregulation of IL-1α and IL-1β expression in Direct(PL) (Fig. 6b). The marker IL-6 was upregulated in cells from Direct(AB + FGF$_{high}$) and there was no expression for IL-8.
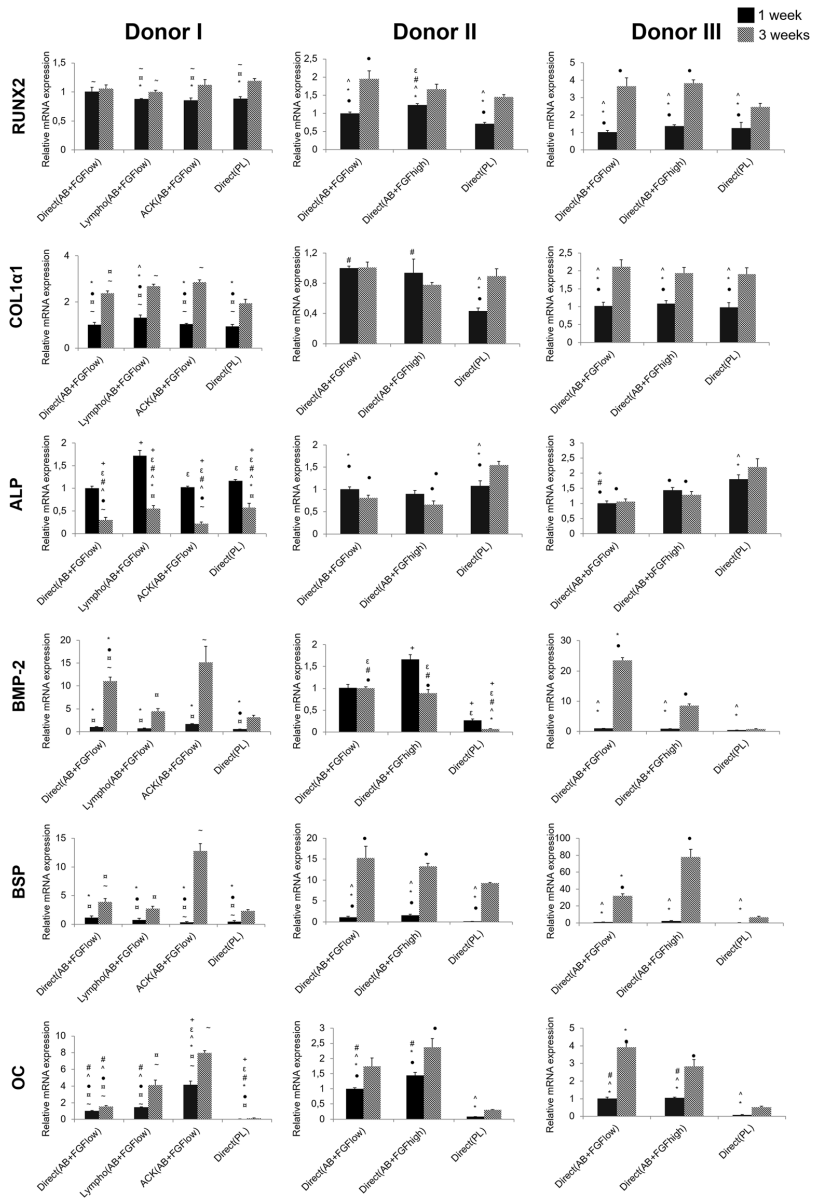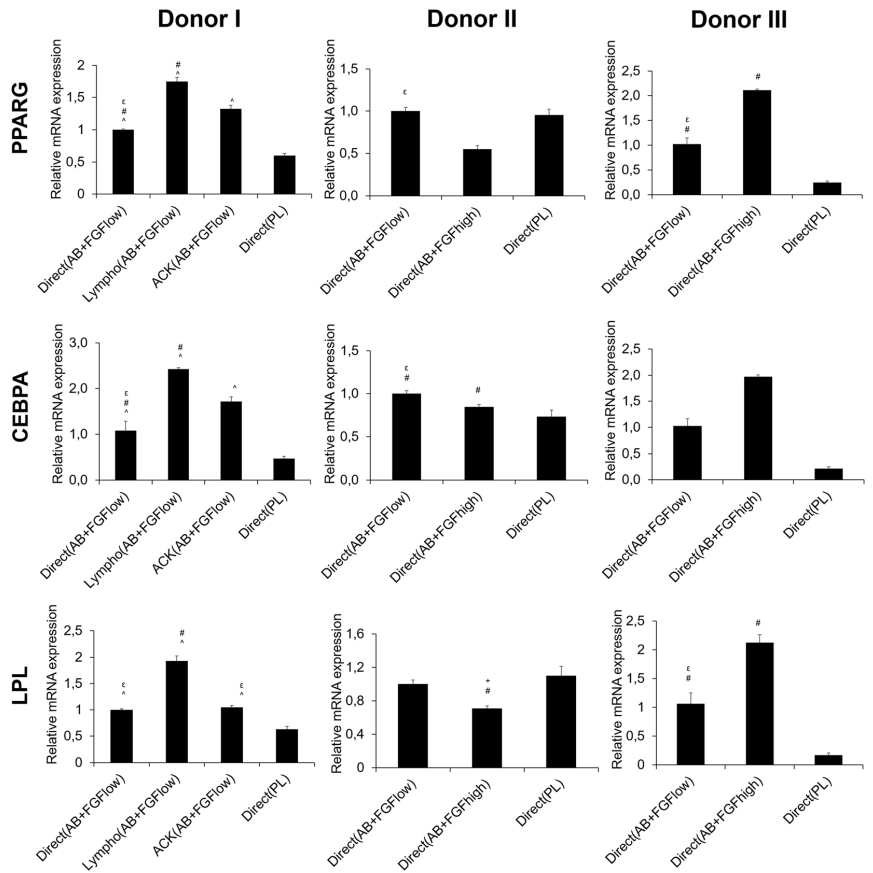
Figure 3. mRNA expressions of osteogenic markers *in vitro* after 1 and 3 weeks post transport. Y axes represent relative mRNA expressions for RUNX2, Col1α1, ALP, BMP-2, BSP and OC. Expression is presented relative to the Direct(AB + FGF$_{low}$) group at 1 week, (p < 0.05).

Histologically, inflammatory cells (neutrophils, plasma cells and lymphocytes) infiltrated all implants in the first week (Fig. 6c red arrows). More inflammatory cells were detected in Direct(AB + FGF$_{high}$) and Direct(PL). Fibroblast-like cells were visible within a dense connective tissue close to the granules in Direct(AB + FGF$_{high}$) and Direct(PL), compared to the interlaced areas of loose connective tissue observed in BCP alone. Thick and organised layers of collagen bordering the granules with cells from Direct(AB + FGF$_{high}$) group (Fig. 6c black arrows)were observed as compared to cells from BCP alone and Direct(PL) group. Peripheral resorption/

Figure 4. mRNA expressions of adipogenic markers *in vitro* after 2 weeks post transport. Y axes represent relative mRNA expressions for PPARG, CEBPA and LPL. Expression is presented relative to the Direct(AB + FGF$_{low}$) group, (p < 0.05).

degradation of the granules was greater in Direct(AB + FGF$_{high}$) compared to the control and to Direct(PL) and cells could be identified within the pores of the granules in Direct(AB + FGF$_{high}$) (Fig. 6c blue arrows).

After 11 weeks *in vivo* multi-nucleated giant cells were seen in close contact to the peripheries of the granules in all implanted groups mostly in the BCP alone and Direct(PL) groups (Fig. 6d black arrows). Integration of the granules with the surrounding tissues appeared to be less obvious, with a decrease in granules' surface area, indicative of accelerated resorption than at 1 week. The granules in Direct(AB + FGF$_{high}$) continued to degrade faster, thus the less visible multi-nucleated giant cells. Granules were situated between areas of dense and disperse connective tissue, with the connective tissue closer to the granules being more dense in the Direct(AB + FGF$_{high}$) and Direct(PL) groups compared to the control. Cellular connective tissue with scattered fibroblast-like cells was more prominent in Direct(PL) (Fig. 6d red arrows).

In vivo osteogenic potential showed variability with different culture conditions. After 1 week there were no expressions for mice osteogenic genes; however the human genes from hBMSC were expressed after implantation. All osteogenic markers evaluated, RUNX2, COL1α2, ALP showed a consistent significant upregulation in the Direct(AB + FGF$_{high}$) group compared to the control and Direct(PL).

Histological sections examined for mineralisation showed that after 11 weeks *in vivo*, the frequency, maturation and quantity of ectopically formed bone were different among the different groups. Cells from Direct(AB + FGF$_{high}$) formed mature mineralised ectopic bone in 83% of the animals, compared to immature mineralised tissues formed in only 17% of the animals implanted with Direct(PL). BCP alone showed no signs of mineralisation, however there were areas of dense collagen seen around the granules. Osteocytes in lacunae were found within the bone like structures in Direct(AB + FGF$_{high}$) (Fig. 7b black arrows). Also, cuboidal osteoblast-like cells were seen surrounding the peripheries of the granules and bordering the newly formed bone.
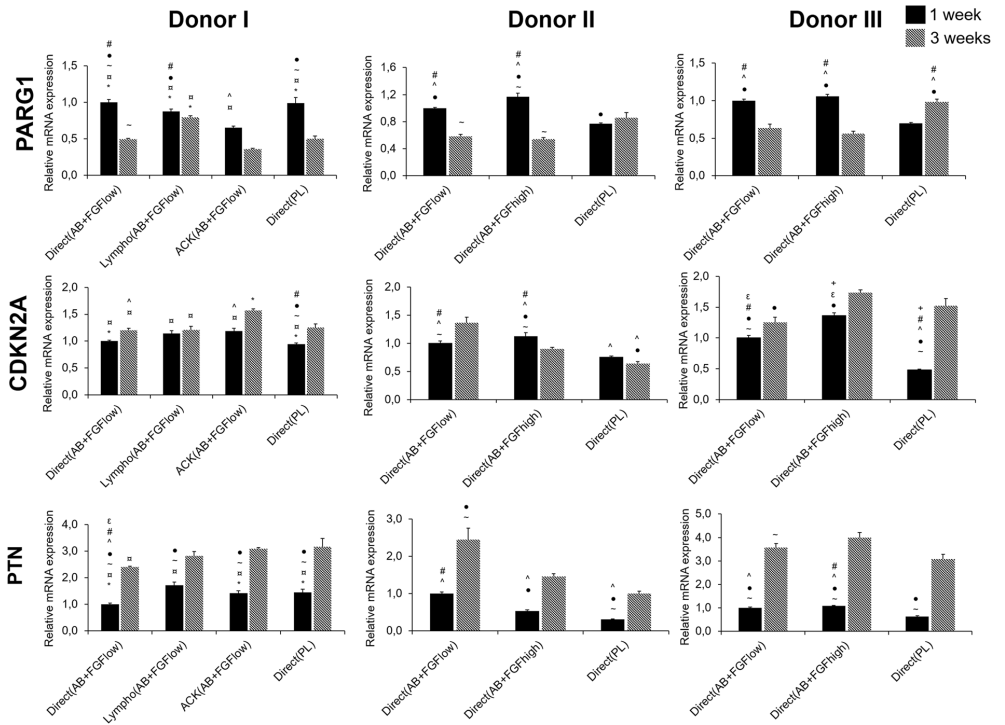
Figure 5. mRNA expressions of replicative senescence genes after 1 and 3 weeks in osteogenic culture. Y axes represent relative mRNA expressions for PARG1, CDKN2A and PTN. Expression is presented relative to the Direct(AB + FGF$_{low}$) group, (p < 0.05).

The Direct(PL) group histologically contained more areas of dense collagen and the de novo-formed bone appeared to have a higher ratio of mineralised immature 'bone-like' regions (Fig. 7b red arrows).

Identifying implanted human BMSC *in vivo*. *In situ* hybridisation using the human-specific repetitive Alu sequence demonstrated that i*n vivo* explants were populated primarily by host connective tissue cells (fibroblasts) depicted by the purple nuclei and purple fibers. Human cells were identified with brown nuclei and were observed after week 1 in both Direct(AB + FGF$_{high}$) and Direct(PL) groups (Fig. 8a black arrows). At week 1, human cells in Direct(PL) were seen to infiltrate a dense connective tissue that is organised and wrapped around the periphery of intact BCP granules. On the contrary, the human cells in Direct(AB + FGF$_{high}$) were observed to populate a looser connective tissue (less organised) that is surrounding BCP granules that are less integrated. A similar picture of loose connective tissue with less human cell population was observed surrounding the less integrated BCP granules in Direct(PL) explants. Morphologically, no differences were observed between the human cells infiltrating in both groups. After 11 weeks human cells were detected in the explants from Direct(AB + FGF$_{high}$) only (Fig. 8b black arrows). The human cells were found embedded in osteocyte lacunae within the mineralised bone formed ectopically with no cells found in the surrounding connective tissue. No inflammatory cells were identified infiltrating the connective tissue or bone which is populated with human cells.

Discussion

In this study, we compared different isolation conditions of MSC from human bone marrow and expansion in different 'humanised' media, specifically, human AB serum (AB) supplemented with FGF2 or PL. Several studies have confirmed that human derivatives can substitute for FBS in expansion of MSC[26,27] and therefore this comparison was not the objective of the current study. Clinical applications of MSC require a cell number that cannot be provided by simple bone marrow aspiration, therefore, an *ex vivo* expansion step is inevitable. However, their clinical applications may be limited by the ability to expand their cell numbers *in vitro* while maintaining their differential potentials and stem cell properties/'stemness'.

In a GMP system, cells are produced under the highest quality requiring product reproducibility[6,7]. Cell survival and quality assurance are essential aspects to be considered in addition to the therapeutic potency. Taking this into consideration, the viability of our cells after shipment was considered comparable to that before shipment, hence successful. However, in some occasions, the GMP facilities are not in close proximity to the clinical
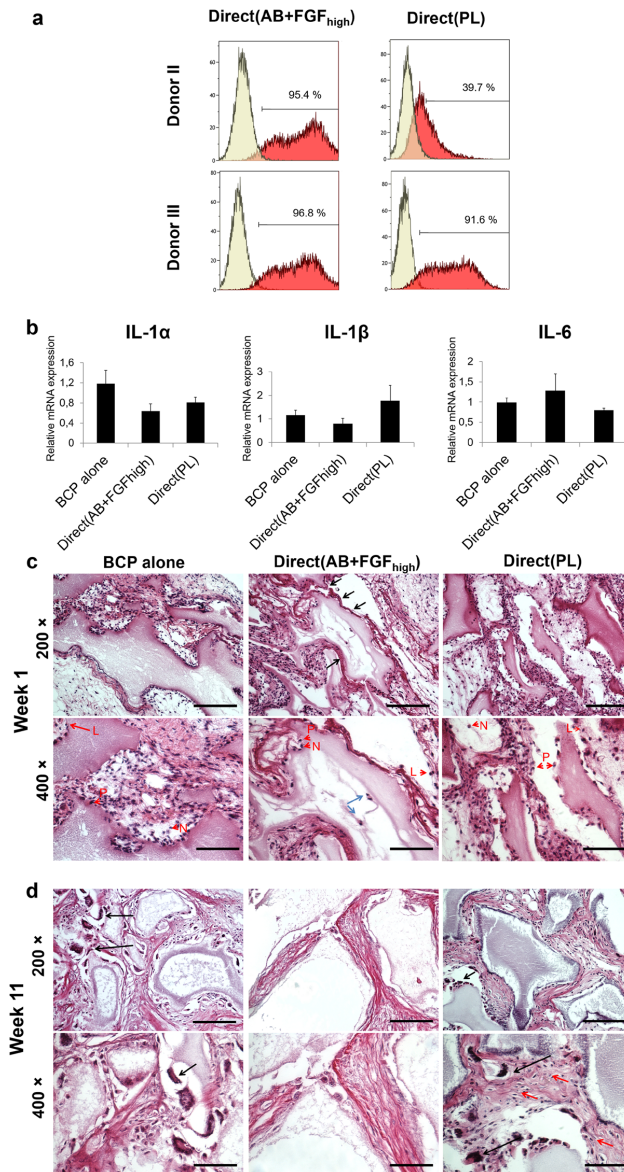
Figure 6. *In vivo* inflammatory and host responses. (**a**) Representative histograms of flow cytometry analysis showing HLA-DR expression on hBMSC pre-implantation; monoclonal antibody control (yellow) and the stained cells (red). (**b**) Mouse relative mRNA expressions 1 week post-implanting of the BCP and cell constructs *in vivo*. Y axes represent mRNA expressions for IL-1α, IL-1β, IL-6 ($p < 0.05$). (**c**) Histological micrographs after 1 week of the different groups implanted. Magnification 400× showing the different inflammatory cells recruited. L - lymphocytes, N - neutrophils, P - plasma cells. (**d**) Histological micrographs after 11 weeks showing recruited foreign body giant cells (black arrows) and a cellular connective tissue with scattered fibroblast-like cells in Direct(PL) (red arrows). Scale 100 μm.

centre where the cell therapy is taking place. The ability to preserve MSC phenotype and function in a sterile condition until the confirmation of an absence of bacterial contamination is important for quality control prior to clinical transplantation[28]. Dimethylsulfoxide (DMSO) and FBS are commonly used as cryoprotectants. However,
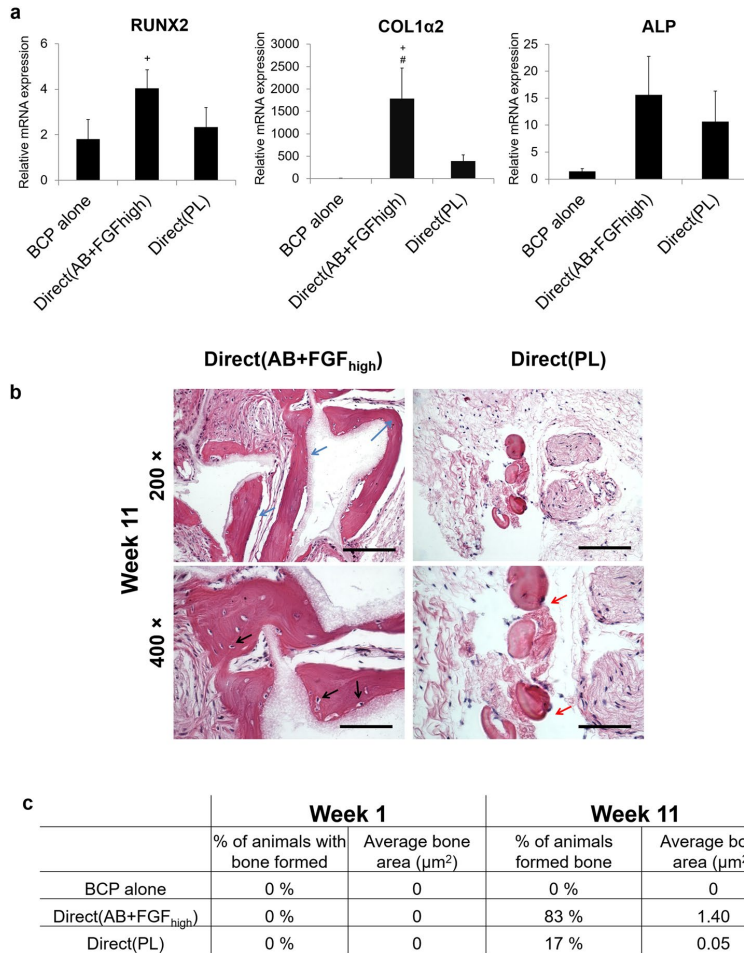
Figure 7. mRNA expression of human osteogenic markers *in vivo* and *de novo* bone formation. **(a)** Relative mRNA expressions after 1 week *in vivo*. Y axes represent relative mRNA expressions for RUNX2, COL1α2 and ALP relative to endogenous reference GAPDH. Expression is presented relative to BCP alone ($p < 0.05$). **(b)** Histological micrographs after 11 weeks showing mature mineralised ectopic bone (blue arrows), osteocyte lacunae in Direct(AB + FGF$_{high}$) explants (black arrows) and immature bone-like regions in Direct(PL) (red arrows). Scale 100 μm. **(c)** Histomorphometrical analyses of the frequency and quantity of bone formed in the different implant groups after 1 and 11 weeks.

the use of DMSO can result in toxicity while animal proteins present in serum-supplemented culture media, can induce immune responses after transplantation. Cryopreserving the MSC in xeno-free can promote cell therapy 'off the shelf', but the cryostorage protocols need to evaluated thoroughly to test the resiliency of the cells. This has been effectively presented with autologous plasma used as a cryoprotectant in combination with DMSO, where it preserved human MSC's therapeutic efficacy after rapid thawing and implantation[28]. However, it has been reported that the cells' behaviour can be affected by different cryoprotectants[29]. Platelet lysate as a cryoprotectant revealed comparable efficacy compared to FBS[30], however how the different types of human-derived cryoprotectants preserve MSC need to be compared in a bone regeneration context. In our results (Table 3), cells expanded in Direct(PL) showed the highest viability in Donor II and III, although this higher viability, compared to the other groups, was not conserved after 24 h of shipment in room temperature. Moreover, we have previously shown that expansion medium supplemented with PL allowed bone marrow MSC to continue proliferating steadily for more than 120 days[31]. Therefore, due to the presence of high amounts of growth factors and cytokines, particularly PDGF in PL[16], which has an anti-apoptotic effect on progenitor cells[32], we could conjecture that PL
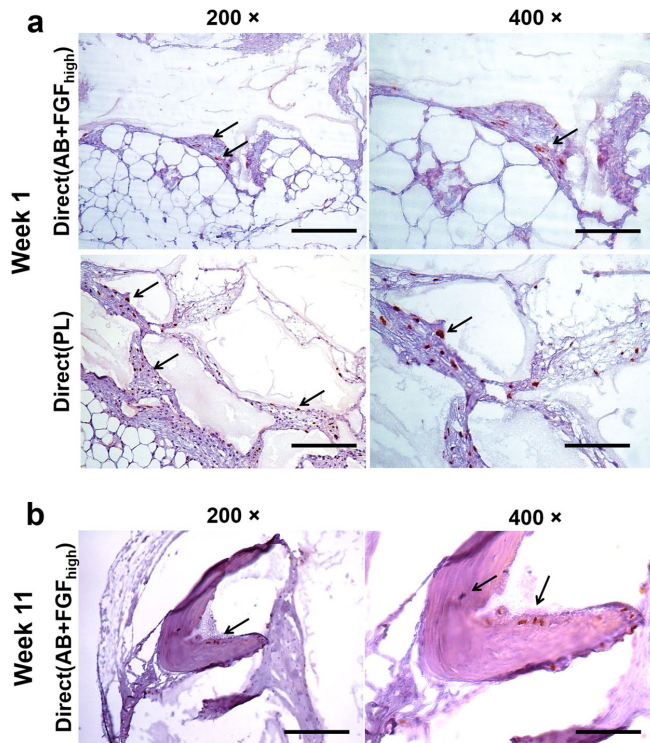
Figure 8. Human cell identification by *in situ* hybridisation of the human-specific repetitive Alu sequence. (**a**) Micrographs showing human cells 1 week post-implantation populated with host cells (purple nuclei) and human cells (brown nuclei) surrounding BCP granules in both Direct(AB + FGF$_{high}$) and Direct(PL) (black arrows). Human cells populating dense connective tissue in Direct(PL) compared to a loose connective tissue in Direct(AB + FGF$_{high}$). (**b**) Human cells identified by the brown nuclei (black arrows) in osteocyte lacunae (black arrows) within the mineralised bone formed in Direct(AB + FGF$_{high}$) after 11 weeks post implantation. Scale 100 μm.

is a cryoprotectant that could preserve the high viability and proliferative capacity expressed during thawing and expansion. However, success of survival after engraftment is not necessarily a similar reflection.

Concerns that the monolayer expansion phase of MSC results in loss of multipotency, the ability to self-renew, and promotes a tendency toward osteogenesis have been previously reported[33]. In our study, hMBCs before differentiation were analysed for presence of pluripotency markers Oct-4 and NANOG, which are reported to function in coherence as key transcription factors for the pluripotent and self-renewing phenotypes of undifferentiated embryonic stem cells[34,35]. However, their role in the self-renewal and undifferentiation of MSC is controversial with postulated mechanisms behind their role in maintaining MSC in an undifferentiated state[36]. Interestingly, the MSC expanded in PL were highly positive for Oct-4 and NANOG mRNA, therefore, PL maintained primitive MSC during expansion, compared with that in AB serum. Similarly, a study using PL showed that MSC preserve their multipotency with elevated expressions of Oct-4 and NANOG compared to when FBS is used to expand MSC[37]. This can be considered advantageous since this will likely affect the functional outcomes following *in vivo* transplantation. Compared with other human supplements, PL was reported to be the human-derived supplement of choice for expanding hBMSC in recent clinical trials[22]. Additionally, CD90, a marker expressed by bone marrow subpopulations of CFU-F[38], was upregulated in Direct(PL) for all donors and least expressed by Direct(AB + FGF$_{low}$) for all donors, which highlights the role played by PL in preserving the MSC multipotency. Nevertheless, many studies have shown that expansion of MSC in different types of media can lead to increased heterogeneity and enrichment of certain subpopulations, which can affect cellular genotypes and phenotypes[39]. Reports have shown that addition of PL to the medium produces MSC with a reduced adipogenic differentiation potential, whereas other studies have shown that it favours osteogenesis and chondrogenesis[40–42]. These conflicting outcomes may be attributed to inconsistency in preparation procedures as well as the effects of PL donors of different ages[43].

Our results also showed variability in the expression of the stemness markers among the groups where hBMSC were expanded in AB + FGF but using different isolation methods. This highlights the additional role played by isolation methods in the cell phenotype/genotype. MSC are isolated from bone marrow using different methods by different laboratories; these include directly plating the whole bone marrow, density centrifugation, red blood cell lysis and magnetic/fluorescence activated cell sorting. In our study, one of the methods used to isolate MSC was processing by density centrifugation, using Lymphoprep density media. In donor I, Lympho(AB + FGF$_{low}$) portrayed the second highest expression for stemness markers after Direct(PL). Density centrifugation separates the mononuclear cell fraction from the other cellular or non-cellular constituents in bone marrow aspirate (*e.g.* red blood cells). The resulting mononuclear cells include a mixed population, such as T cells, B cells monocytes, hematopoietic stromal cells and MSC. The density centrifugation process itself reduces the total yield of mononuclear cells from bone marrow[44], but may increase the colony forming unit fibroblasts (CFU-F) efficiency compared to direct plating/seeding and ACK lysis methods[45]. This can explain their higher expressed stemness. Inter-donor variations cannot be avoided, however, to rule out variations that have been demonstrated when comparing the phenotype of MSC population derived from two serial bone marrow aspirates from the same person[46], the same bone marrow sample was divided and processed differently in this study.

In our AB serum, it was supplemented with FGF2, and purified growth factors, such as FGF2, are added to culture conditions even in the presence of FBS or blood-derived supplements to enhance proliferation and overall behaviour[47]. FGF2 is a known mitogen of MSC, also functioning to maintain multipotency and to promote subsequent differentiation *in vitro*[47,48]. However, their effects can greatly vary depending on their concentrations and interactions, as portrayed by our results. The concentration of FGF-2 was doubled, but that increase was not reflected directly on the expression of osteogenic genes for example. Concentrations, not standard, up to 10 ng/ml of FGF2 are reported in the literature, causing difficulties when attempting to compare studies.

In vitro inflammatory gene expressions were evaluated in undifferentiated hBMSC to evaluate the role played by the isolation and expansion conditions solely. The pro-inflammatory marker IL-1α was least expressed in Lympho(AB + FGF$_{low}$). This method compared to others involves the selection of the bone marrow mononuclear cells and it is in line with a previous report where directly seeding complete bone marrow or using methods not involving selecting mononuclear cells preserved the immunomodulatory capacity of MSC[49]. IL-6 and IL-8 are inflammatory cytokines that play important roles in osteogenic differentiation for bone regeneration and remodelling[50,51]. Our results showed the highest expression of IL-6 from Direct(PL) in donor I and the second highest in donor II and III. The secretion of IL-6 was shown to be increased by MSCs cultured with PL compared with FBS[52] and IL-6 has been reported to maintain MSC stemness[53]. Considering that Direct(PL) cells showed a relatively late osteogenic differentiation, this explains the reverse expressions of IL-8, since in bone regeneration this marker is involved with osteoclast function which is in late stages of remodelling[50]. MSC have the ability to express HLA-II when exposed to inflammatory stimulants[54] and these stimulations trigger their immunosuppressive function[55]. HLA-II antigens are recognised by CD4 + T lymphocytes, and MSC avoid immune rejection by immunomodulation of the local environment[56]. HLA-DR is an MHC class II cell surface receptor, and a stronger expression of HLA-DR compromises the immune privilege of MSC[57]. Direct(PL) cells showed a lower expression of HLA-DR compared to Direct(AB + FGF$_{high}$), which correlates with the more 'stemness' phenotype expressed from Direct(PL) cells and their higher expression of anti-inflammatory marker IL-6 *in vitro* and reduced pro-inflammatory markers (IL1α and β). It has been reported that the HLA-II expression in MSC displays an inversely proportionate relation with the stemness of the cells[58].

Nevertheless, the impact of HLA-DR expression on MSC potency and function remains controversial due to its dynamic expression from MSC in culture with time[56]. Different culture conditions (human serum versus platelet lysate) were not found to affect the HLA-DR expression from BMSC[56], however addition of FGF was reported to express higher HLA-DR in BMSC compared to when cells were cultured in platelet lysate[59], in line with our results. The expression of HLA-DR has been suggestive of differentiation commitment[56], which can explain the osteogenic potential of cells from AB + FGF *in vitro* and *in vivo*.

In vivo, when the hBMSC were implanted, no human inflammatory markers were detected at mRNA level, however the implanted cells posed varying inflammatory reactions from the host cells between groups.

Our *in vitro* osteogenic differentiation results showed mostly elevated expressions of osteogenic markers when cells were generally expanded in AB + FGF2. It has been reported previously that MSC cultured in FBS in combination with FGF-2 showed a superior growth compared to PL[49]. However, a closer look into when the different isolation methods were compared in donor I, a significant difference was portrayed even when the cells were expanded in the same media (AB + FGF2). The bone markers BMP-2, BSP and osteocalcin showed significantly higher expressions after 3 weeks in the ACK(AB + FGF$_{low}$). This can be attributed as well to the fact that other isolation methods, such as density centrifugation increase the sub-population of naive MSC more prone to self-renewal than differentiation[45].

MSC expanded in PL showed relatively higher expressions in the early bone markers, such as RUNX2 and ALP. This compliments the high stemness markers that were expressed which propose the late differentiation of the cells in this condition. A previous study reported that addition of FGF-2 in the culture medium resulted in reduction in expression of ALP[60], which could be a postulation for our higher ALP expressions from Direct(PL) in donor II and III. Besides the naive MSC state in the Direct(PL), another factor for the lesser osteogenic potential could be the addition of heparin in the medium to prevent gel formation. It was shown that a relatively high concentration of heparin in culture media supplemented with human PL compromises proliferation as well as adipogenic and osteogenic differentiation of MSC[61].

Characterising hBMSC performance and potency as a therapeutic entity requires additional tests for *in vivo* differentiation potential. Based on the *in vitro* results, the direct seeding method was chosen to go further. This helps to reduce confounding factors and establish methods easy to standardise with least manipulation for the

bone marrow aspirates. As previously reported, MSC expanded in FBS are able to undergo bone formation when implanted on BCP scaffolds[62], however it is important to define that this characteristic and quintessential multipotent MSC phenotype are not lost with the optimised humanised culture conditions proposed in this study.

In vivo, hBMSC expressed the osteogenic genes (RUNX2, COL1α2 and ALP) when expanded in Direct(AB + FGF$_{high}$) and showed a higher frequency of bone formation too, which reflects cell survival and differentiation, indicating a direct contribution of the implanted hBMSC in bone formation. Interestingly, the *de novo* bone in Direct(PL) seemed to have immature regions with more disorganised collagen, suggestive of woven bone morphology and indicating tissue-formation processes were still continuing after 11 weeks of implantation. This can be reflected on the higher self-renewing potential of the cells in this group that was depicted *in vitro*. When observed closely, the collagen of the new bone in the Direct(AB + FGF$_{high}$) group appeared much more organised with residing osteocytes within lacunae, suggestive of lamellar structure. Several studies suggest a direct contribution of MSC to regenerate bone as they differentiate into osteoblastic lineage when implanted locally, and contrary studies postulate that the implanted MSC exert a paracrine effect on host cells thus home circulating hematopoietic progenitors[63] and endogenous osteogenic progenitor cells[64]. The *in situ* hybridisation results suggest the cells from the different groups might have contributed differently when forming bone. In Direct(AB + FGF$_{high}$) human cells were still identified after 11 weeks, located in the lacunae of mature mineralised bone, which was not the case for Direct(PL). It could be that the less differentiated cells from Direct(PL) released cytokines and growth factors to recruit endogenous progenitor cells. Although no human cytokines were detected from the explants, however the mouse pro-inflammatory markers detected were highly expressed in this group. Therefore, the host inflammatory environment induces the recruitment and the homing of MSC[65]. IGF-1 and PDGF that are reported to play a role in homing MSC[66] are major constituents of PL[16].

Replicative senescence is reflected by significant morphological changes; cellular enlargement, debris and vacuoles intracellulary leading to a cease of proliferation[67]. The cells in our study were expanded for only 2 passages before they were used in the *in vitro* and *in vivo* experiments. No senescent/related morphological changes or phenotypical changes were identified when the cells were routinely observed macroscopically. However, previous reports have shown that replicative senescence of MSC is a continuous process starting from the first passage[67], and this process includes, in addition to alterations in phenotype and differentiation potential, senescence-associated gene expression changes. Therefore, to reflect the heterogeneity in the cellular aging process, global gene expression patterns have been evaluated and still constantly identified. PTN demonstrated to play important roles in survival and self-renewal of human embryonic stem cells and retention of bone marrow hematopoietic stem cells. Its down-regulation was shown to be associated with decline of proliferation capacity of senescent MSC[68]. The mRNA expression of PTN after 3 weeks with a significantly higher upregulation in the AB + FGF groups in general in donor II and III could elucidate the survival of Direct(AB + FGF$_{high}$) *in vivo* up to 11 weeks compared to cells cultured in Direct(PL). The trend was reflected in the other markers, PARG1 and CDKNA2, cell cycle inhibitors that were downregulated in Direct(PL) in most donors in week 1. Replicative senescence-associated gene expression changes in hBMSCs isolated in different methods and expanded under xenogenic and xeno-free culture conditions demonstrated high similarity[68]. From our results, an effect of culture conditions on the senescence of the cultured hBMSC could be detected and an explanation for the *in vivo* cell survival and *in vivo* performance of the different groups can be postulated. However, several factors can be considered that influence the variation on gene expressions *in vitro*, such as donor age and cell density.

In addition to the isolation methods, the differing behaviours of MSC can be ascribed to the different cytokine contents of AB serum and PL. Also this might mean that 10% supplement of each is not a standard comparison as it may seem. It has been documented, for example, that age-related differences in human serum and PL composition occur and have a direct effect on MSC performance[43]; however, our results are related to commercially available allogeneic serum pooled from many hundred donors and PL pooled from 76 donors. A combination of human serum and PL to supplement culture medium showed an improved proliferation of MSC compared to when expanded in only human serum[21].

In our results, Direct(AB + FGF$_{high}$) cells expressed superior osteogenic potential *in vivo*, and these cells were identified until 11 weeks *in vivo*. This indicates that the poor survival of transplanted cells is a limitation for clinical regeneration that depend on long-term engraftment. A better understanding of how the cytokines present in inflammatory environments *in vivo* moderate MSC could be useful to develop more effective priming strategies to enhance MSC survival and subsequent therapeutic efficacy.

Coupling GMP xeno-free MSC production with the manufacturing of primed MSC can be considered as complementary strategies to enhance cell survival and therapeutic efficacy. Potential mechanisms of MSC primed therapeutics include gene editing[69] to engineer MSC and thus promoting tissue regeneration through cell differentiation[70]. Moreover, via the delivery of bioactive factors through different secretory modes including *ex vivo* cell engineering[71] or the production of induced MSC from induced pluripotent stem cells and preserve immunomodulatory characteristics in addition to enhanced cell survival and proliferation[57].

Conclusions

To fully recognise and exploit the therapeutic potential of MSC, an inclusive evaluation of their stemness, lineage, cell surface markers and transcription factors, in line with their isolation and expansion was required. In our work, we evaluated the effects of isolation methods and 'humanised' culture conditions on the potency of bone marrow derived MSC. Taken together, our results showed a significant effect of the isolation method and demonstrated a relatively consistent pattern of efficacy from 3 donors, and portrayed a tendency of hBMSC expanded in PL to retain a more stem-like phenotype which elucidates their delayed differentiation and different inflammatory expressions.

Data availability
Data will be made available on request.

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Author contributions

S.S. contributed to conception and design, performing the *in vitro* and *in vivo* experiments, data analyses, interpretation and manuscript writing. H.R.W.A. contributed to *in vivo* experiments and PCR analyses. T.A.K. and J.E.B. contributed to conception and design, isolation of cells, flow cytometry and data interpretation. J.A. and P.L. contributed to *in situ* hybridisation and data interpretation. S.M.A. contributed to *in vitro* and *in vivo* experiments. D.E.C. contributed to conception and design, histological analyses and data interpretation. K.M. contributed to conception and design and data interpretation. All authors read and approved the final manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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Paper II

Xeno-free generation of human induced pluripotent stem cells from donor matched fibroblasts isolated from dermal and oral tissues

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II

RESEARCH

Open Access

Xeno-free generation of human induced pluripotent stem cells from donor-matched fibroblasts isolated from dermal and oral tissues

Hassan R. W. Ali[1], Salwa Suliman[1], Tarig Al-Hadi Osman[1], Manuel Carrasco[2,3], Ove Bruland[4], Daniela-Elena Costea[2,3,5], Helge Ræder[6,7*†] and Kamal Mustafa[1*†]

Abstract

Background Induced pluripotent stem cells (iPS) can be generated from various somatic cells and can subsequently be differentiated to multiple cell types of the body. This makes them highly promising for cellular therapy in regenerative medicine. However, to facilitate their clinical use and to ensure safety, iPS culturing protocols must be compliant with good manufacturing practice guidelines and devoid of xenogenic products. Therefore, we aimed to compare the efficiency of using humanized culture conditions, specifically human platelet lysate to fetal bovine serum, for iPS generation from different sources, and to evaluate their stemness.

Methods iPS were generated via a platelet lysate or fetal bovine serum-based culturing protocol from matched dermal, buccal and gingival human fibroblasts, isolated from healthy donors ($n = 2$) after informed consent, via episomal plasmid transfection. Pluripotency, genotype and phenotype of iPS, generated by both protocols, were then assessed by various methods.

Results More attempts were generally required to successfully reprogram xeno-free fibroblasts to iPS, as compared to xenogenic cultured fibroblasts. Furthermore, oral fibroblasts generally required more attempts for successful iPS generation as opposed to dermal fibroblasts. Morphologically, all iPS generated from fibroblasts formed tight colonies surrounded by a reflective "whitish" outer rim, typical for iPS. They also expressed pluripotency markers at both gene (*SOX2*, *OCT4*, *NANOG*) and protein level (SOX2, OCT4). Upon stimulation, all iPS showed ability to differentiate into the three primary germ layers via expression of lineage-specific markers for mesoderm (*MESP1*, *OSR1*, *HOPX*), endoderm (*GATA4*) and ectoderm (*PAX6*, *RAX*). Genome analysis revealed several amplifications and deletions within the chromosomes of each iPS type.

Conclusions The xeno-free protocol had a lower reprogramming efficiency compared to the standard xenogenic protocol. The oral fibroblasts generally proved to be more difficult to reprogram than dermal fibroblasts. Xeno-free dermal, buccal and gingival fibroblasts can successfully generate iPS with a comparable genotype/phenotype to their xenogenic counterparts.

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Introduction

Mesenchymal stem cells (MSC) have long been instrumental in regenerative medicine due to their multipotency, ability to self-renew and high proliferative capacity [1–3]. Promising results utilizing MSC for bone regeneration in both preclinical and clinical settings have been reported recently [4–9]. However, due to a number of challenges surrounding MSC, the quest to exploring alternative sources, such as pluripotent cells, is crucial [10–12]. Embryonic stem cells (ESC) can be expanded indefinitely without undergoing replicative senescence or aging due to their high telomerase expression. The main property that sets ESC apart from other cells is their pluripotent nature, meaning they can give rise/differentiate to cells of the three primary germ layers (mesoderm, endoderm and ectoderm) [13]. With time, however, concerns were raised surrounding the use of ESC due to drawbacks associated with the isolation process, immunogenicity and risk of teratoma formation [13–15]. The discovery of induced pluripotent stem cells (iPS), artificially generated via genetic alteration/reprogramming of mature somatic cells, offered some advantages relative to ESC [16–18]. Similar to ESC, iPS express pluripotent markers, have unlimited proliferation potential and possess the ability to differentiate into the three primary germ layers [19, 20]. Furthermore, iPS can be generated autologously and/or from a selected genetic background [15, 19]. To gain pluripotency, the reprogramming process typically involves transfecting adult somatic cells with certain pluripotency markers. Yamanaka initially reprogrammed fibroblasts by using four transcription factors, OCT4, SOX2, KLF4 and c-MYC, also known as the OSKM factors, or the Yamanaka factors [16, 17]. Since then, other groups have successfully reprogrammed adult cells to iPS via the use of various different cocktails of transcription factors [18, 21]. A multitude of methods for delivering these factors have been developed, which include both integrating and non-integrating methods. Integrating methods, such as retro and lentiviral delivery methods, leave behind an undesirable footprint via integration of exogenous genetic material into the cell genome. Therefore, in order to make iPS more clinically applicable, non-integrating reprogramming methods have been developed with zero footprint [21, 22]. These non-integrating methods include, among others, the use of episomal plasmids for delivery. Studies have shown that the episomal vectors do not persist and are spontaneously lost after a period

of time [21, 23]. Nevertheless, despite the absence of any signs of the original plasmids, other footprints are possible, including artificially introduced genetic alterations. Copy number variations (CNV), such as deletions, insertions and duplications, exist naturally as structural variants in the human genome [24]. Such sub-chromosomal aberrations, along with whole-chromosome aneuploidies, have been regularly reported in human pluripotent cells (both ESC and iPS), with iPS likely having a higher number of CNVs than ESC [25–27]. This could be due to the reprogramming process itself being associated with increased CNV levels in the early stages of the resultant iPS. The number and total size of these CNVs, however, decrease dramatically with continued propagation/passaging of iPS [28]. Fibroblasts, specifically dermal fibroblasts, were the first cell type to be utilized for iPS generation [17]. Since then, different somatic cells have been used to generate iPS including keratinocytes, blood cells, dental pulp stem cells and mesenchymal stem cells [29–33]. Fibroblasts remain the most commonly used cell type for iPS generation, as they are generally easy to obtain and handle, and are commercially available for research purposes. Various cell types from the oral cavity have been used for the generation of iPS, as cells can be easily collected during dental procedures, without the need for extra invasiveness. In addition, wounds in the oral cavity heal rapidly without scar formation and with minimal patient discomfort [34, 35]. Hence, transitioning to oral sources for iPS generation has the potential to be a valuable approach.

To comply with good manufacturing practice (GMP) guidelines, it is vital to create cell culture protocols that are safe and standardized. Currently, media supplemented with fetal bovine serum (FBS) is the most widely used method of cell expansion [36]. Despite its large scale and frequent use, FBS is associated with a multitude of ethical, scientific and safety issues. For instance, variations in serum composition result in batch-to-batch heterogeneity, causing morphological and phenotypical differences, ultimately affecting reproducibility of cell expansion protocols [37–39]. As a result, FBS production has come under great scrutiny, and there is an increasing demand and need for animal-free culture techniques, which would allow for a safer and more ethical practice. With that being said, xeno-free alternatives have been developed, and platelet lysate (PL) has emerged as a promising "GMP compliant" candidate to replace FBS. PL is typically prepared

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from platelet derivatives which contain and release high concentrations of growth factors. These growth factors aid in the expansion of cells in culture. Moreover, PL is generally pooled from multiple donors, reducing donor-based variations [40, 41]. These attributes make PL an attractive alternative to FBS as a supplement to expansion media.

A few studies have claimed to generate iPS from xeno-free conditions, yet their protocol either includes FBS media for the culture of the somatic cells prior to iPS generation, or they fail to mention the somatic cell culture method altogether [42–44]. As far as we know, only a handful of studies have previously generated iPS using an entirely xeno-free protocol [45–48]. However, no effort was made to compare iPS generated from xeno-free protocols to those generated from xenogenic ones. Due to the aforementioned challenges, it is important to implement protocols that comply with GMP guidelines, *i.e.*, xeno-free culturing protocols, while also utilizing easily accessible sources for iPS generation. Therefore, in this study we aimed to analyze and compare iPS generated from donor-matched fibroblasts from different sources and evaluate their stemness. Furthermore, the study was aimed at evaluating and comparing the efficiency of using humanized culture conditions, specifically PL to FBS, on the generation of potent iPS.

Materials and methods
Fibroblast source and culture
Dermal, buccal and gingival samples were acquired from two healthy voluntary donors (Donor 1 (D1); female aged 40–50, and donor 2 (D2); female aged 50–60), following informed consent. All three samples were collected from each donor; dermal samples were obtained from the anterior forearm, buccal samples from the inside of the cheek and gingival samples from the gingiva above the upper first molar. The specimens were transported in Dulbecco's modified Eagle's medium (DMEM) (Gibco, ThermoFisher Scientific, Massachusetts, USA) supplemented with 3% penicillin/streptomycin (GE, Healthcare) and immediately processed for fibroblast isolation. Briefly, fibroblasts were isolated via the enzymatic digestion protocol as previously described [49]. Following isolation, fibroblasts were cultured and expanded in DMEM supplemented with 5% PL (Blood Bank, Haukeland University Hospital, Bergen, Norway) or 10% FBS (Gibco, ThermoFisher Scientific, Massachusetts, USA, catalog number: 10270106), creating two different culture conditions for cell expansion. The cells cultured in FBS were supplemented with 1% penicillin/streptomycin (GE, Healthcare), while the cells in PL were supplemented with 1% penicillin/streptomycin and heparin at a concentration of 2 IU/ ml (LEO Pharma). The morphology of the cells was observed using Nikon's Inverted Light Microscope ECLiPSE Ts2R-FL (NIKON INSTRUMENTS EUROPE B.V., Amsterdam, the Netherlands). The fibroblast cell lines were regularly checked for mycoplasma contamination, and all lines were free of contamination prior to transfection.

Fibroblast reprogramming and iPS culture
Approximately $5—6\times10^5$ cells (passage 7–10) were transfected (Nucleofector 2b Device, Lonza, Switzerland) with 1 μg of each of the three episomal reprogramming plasmids (*pCXLE-hOCT3/4-shp53*, *OCT4 & shRNA p53*; *pCXLE-kSK*, *SOX2 & KLF 4*; *pCXLE hUL*, *L-MYC & LIN28)* and plated onto a six-well plate containing either FBS or PL supplemented DMEM (Gibco, ThermoFisher Scientific). Upon reaching confluency, the cells were passaged onto a 10 cm dish precoated with Geltrex (Gibco, ThermoFisher Scientific). The following day, culture media was changed to StemFlex media (StemFlex Medium, Gibco, ThermoFisher Scientific). Media was then changed every 1–2 days until stable colonies began to appear. Colonies were deemed stable upon formation of compact, round colonies with distinct borders [50]. Three colonies were then transferred to a Geltrex (Gibco, ThermoFisher Scientific) coated well in a 24-well plate, one colony per well. Each iPS colony was cultured individually in StemFlex media (StemFlex Medium, Gibco) and considered to be a biological replica. Gentle Cell Dissociation Reagent (Stem Cell Technologies, Vancouver, Canada) was used for cell detachment for passaging. Characterization and analyses of iPS were performed after passage 15. Cell morphology was observed using Nikon's Inverted Light Microscope ECLiPSE Ts2R-FL (NIKON INSTRUMENTS EUROPE B.V., Amsterdam, the Netherlands). The iPS were regularly checked for mycoplasma contamination, and all lines were free of contamination prior to analysis.

iPS characterization
Trilineage differentiation
The iPS were subjected to directed differentiation using STEMdiff Trilineage Differentiation Kit (Stem Cell Technologies, Vancouver, Canada). Briefly, iPS were detached from well plates using Gentle Cell Dissociation Reagent (Stem Cell Technologies) and centrifuged at 300 g for 5 min. The pellet was then resuspended in 1 ml of Single Cell Plating Medium according to the manufacturer's instructions. The cells (8×10^5 cells/well for endoderm and ectoderm differentiation and 2×10^5/well for mesoderm differentiation) were transferred to a Geltrex coated 12-well plate. The media was then changed daily for 5 days (mesoderm and endoderm) and 7 days (ectoderm).

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iPS Gene expression analysis (RT-PCR)

The pluripotency of the iPS along with their ability to differentiate into the three primary germ layers was assessed via quantitative real-time PCR (qPCR). Total RNA was isolated using a tissue RNA isolation kit (Maxwell, Promega, WI, USA), and a NanoDrop spectrophotometer (ThermoScientific, Delaware, USA) was used to check the quantity and purity of the isolated RNA. Total RNA (300 ng) was reverse transcribed, according to the manufacturer's instructions, using a high-capacity complementary DNA reverse transcription kit (Applied Biosystems, CA, USA). qPCR was performed on a StepOne Plus system, using TaqMan gene expression assays (Applied Biosystems), to quantify the gene expression of pluripotency markers (*SOX2, OCT4, NANOG*) and trilineage markers for mesoderm (*MESP1, OSR1, HOPX*), endoderm (*GATA4*) and ectoderm (*PAX6, RAX*) lineages. Data were analyzed using the *ΔΔCt* method. Gene expression was normalized to that of the housekeeping gene, *GAPDH*. Expression of pluripotency markers is presented as fold changes relative to the control, dermal fibroblasts (DF) in FBS. Expression of trilineage markers is presented as fold changes relative to uninduced DF-iPS in FBS. An overview of the primers used for the gene expression analysis is presented in Additional file 1.

Flow cytometry

The iPS phenotype was analyzed via flow cytometry for specific markers, namely SOX2 and OCT4 (BD Biosciences, San Jose, CA, USA), according to the manufacturer's instructions. The cells ($\sim 5 \times 10^5$) were fixed in 10% buffered formalin, for 10 min, and permeabilized via 0.1% Triton X, 15 min in the dark. The pellet was then blocked in 0.5% bovine serum albumin, BSA (Sigma-Aldrich, St. Louis, MO, USA), in phosphate-buffered saline (PBS), for 10 min at room temperature. Conjugated monoclonal antibodies were then added to the pellet, and the cells were incubated in the dark for 30 min at 4 °C. The cells were then washed with PBS before being resuspended in PBS. Stained samples were analyzed and compared to the corresponding unstained samples (negative control). The final quantification was performed with a BD Accuri flow cytometer (BD Biosciences), and the data were analyzed using FlowJo (FlowJo, LLC, Ashland, OR, USA).

Chromosome microarray analysis

Whole-genome high-resolution chromosome microarray analysis was performed using the Applied Biosystems CytoScan HD Array Kit and Reagent Kit Bundle (Applied Biosystems Catalog number: 901835) according to the manufacturer's protocol. Briefly, 250 ng of genomic DNA was digested with the restriction enzyme NspI and then ligated to an adapter, followed by PCR amplification using a single pair of adapter primers. The PCR products were purified using magnetic beads (Agencourt AMPure, Beckman Coulter, Beverly, MA). Purified PCR products were then fragmented using DNase I, and the fragmented PCR products end-labeled with biotin and then hybridized to the array using the Affymetrix GeneChip Hybridization Oven 645 (Affymetrix Inc., USA). Arrays were washed and stained using a GeneChip Fluidics Station 250 and scanned using an Affymetrix GeneChip Scanner 3000 7G (Affymetrix Inc.). Scanned data files were generated using Affymetrix GeneChip Command Console Software, version 4.1, and analyzed with Affymetrix Chromosome Analysis Suite version 4.2.1 (ChAS) (Affymetrix Inc.) and BENCH Lab CNV—version 5.1.12 (Agilent Technologies, USA). Filtration was performed against a list of common abbreviations acquired from Affymetrix. Duplications were filtered if at least 90% overlap, containing at least 80 markers and listed at least 25 times in the list of common abbreviations. Deletions were filtered if 90% overlap, containing at least 30 markers and listed at least 25 times in the list of common abbreviations. LSCH regions less than 5 Mbp or supported by less than 500 markers were filtered.

Ethical approval

Approval was granted by the Ethical Committee for Medical and Health Related Research in West Norway (REK 80005). Tissue samples were collected from two healthy voluntary donors after obtaining informed consent.

Data presentation and statistical analysis

Statistical analysis was performed via IBM SPSS Statistics (SPSS Inc.). Data are presented as mean values (±standard deviation). Statistical significance was determined via an independent samples T-test when comparing two groups and one-way analysis of variance (ANOVA) when comparing more than two groups. A *p*-value of < 0.05 was considered statistically significant.

Results

Isolation of fibroblasts in xeno-free conditions

The isolated matched dermal, buccal (BF) and gingival (GF) fibroblasts displayed a spindle-shaped morphology (Fig. 1).

Generation of xeno-free iPS

Matched DF, BF and GF from 2 donors were reprogrammed into iPS. The protocols for generating iPS from the different fibroblasts are illustrated in Fig. 2. The maximum number of reprogramming attempts was set at nine, after which the success rate (based on the number of attempts required to successfully develop iPS colonies) was determined. All the iPS reprogrammed successfully
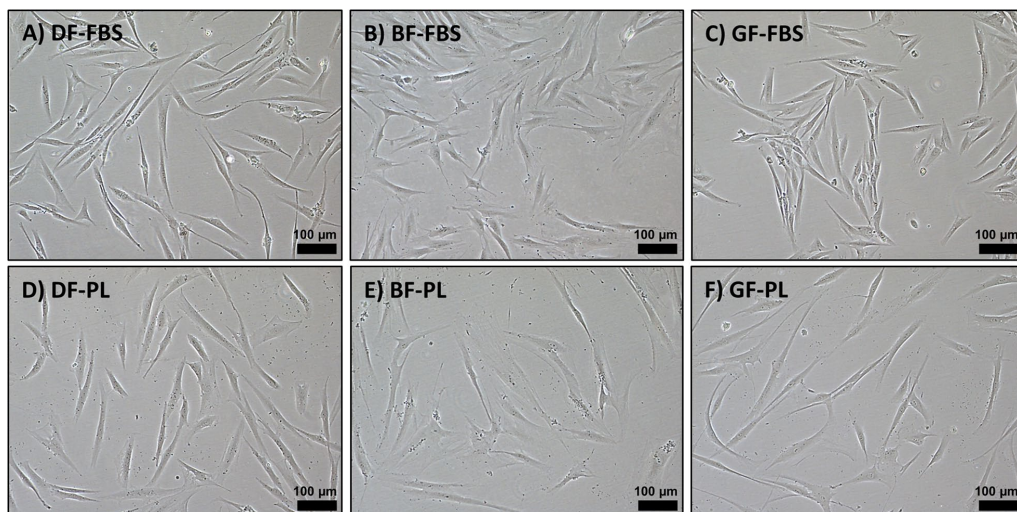
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Fig. 1 Representative light microscopy images demonstrating the morphology of the fibroblasts from different sources expanded in FBS (A–C) and PL (D–F). Scale bar: 100 µm (DF: dermal fibroblasts, BF: buccal fibroblasts, GF: gingival fibroblasts, FBS: fetal bovine serum, PL: platelet lysate)

with the exception of one sample of gingival fibroblasts isolated in PL (GF-PL, D2). Fibroblasts isolated and grown in FBS generally showed a higher reprogramming success rate than those in PL. In most cases, DF showed the highest reprogramming success rate, while BF and GF showed varying success rates with no clear pattern (Table 1). Due to the BF-PL from D2 not yielding any iPS, the characterization of the iPS in this study was limited to the those obtained from D1.

Stemness of the generated iPS
All the reprogrammed fibroblasts developed a stable colony morphology resembling ESC [50]. Morphologically, the cells grew in colonies, surrounded by a reflective "whitish" border, that increased in size as the cells proliferated, ultimately fusing with other colonies in the same well (Fig. 3).

Generated iPS express pluripotent genes
Gene expression analysis showed that the iPS expressed significantly higher levels ($p < 0.001$) of the pluripotency markers, *SOX2*, *OCT4* and *NANOG*, than their respective controls (undifferentiated fibroblasts) which showed little to no expression of these markers (Fig. 4). The iPS-FBS generally displayed higher levels of these pluripotency markers than their matched iPS-PL (Fig. 4).

Within the iPS-FBS, the GF-iPS revealed higher expression of *SOX2* and *NANOG* than both BF-iPS (significantly higher) and DF-iPS (significantly higher in the case

of *NANOG*). Expression of *OCT4* by the iPS-FBS was comparable. The DF-iPS in turn revealed higher expression of *SOX2* (significantly higher) and *NANOG* than the BF-iPS, and slightly higher expression of *OCT4* than both the oral iPS. Within the iPS-PL, the GF-iPS showed a significantly higher expression of *OCT4* and *NANOG* than both the DF-iPS and BF-iPS, which in turn showed comparable expression. *OCT4* expression was also significantly higher in the BF-iPS when compared to the DF-iPS. *SOX2* expression was significantly higher in the DF-iPS than both the BF-iPS and GF-iPS (comparable).

Proteomics of pluripotency markers
Flow cytometric analysis generally revealed SOX2 and OCT4 positive iPS-FBS and iPS-PL. Analysis of the iPS-FBS revealed > 97% of cells positive for SOX2 and > 91% positive for OCT4. Analysis of the iPS-PL revealed > 97% of cells positive for SOX2 and > 94% positive for OCT4 (Fig. 5).

Trilineage differentiation ability of iPS
Upon stimulation, all iPS showed ability to differentiate into the three germ layers, via expression of lineage-specific markers for mesoderm (*MESP1*, *OSR1*, *HOPX*) (Fig. 6A–C), endoderm (*GATA4*) (Fig. 6D) and ectoderm (*PAX6*, *RAX*) markers (Fig. 6E, F). Induced iPS showed significantly ($p < 0.05$) higher expression of the trilineage markers than their respective controls (undifferentiated iPS).
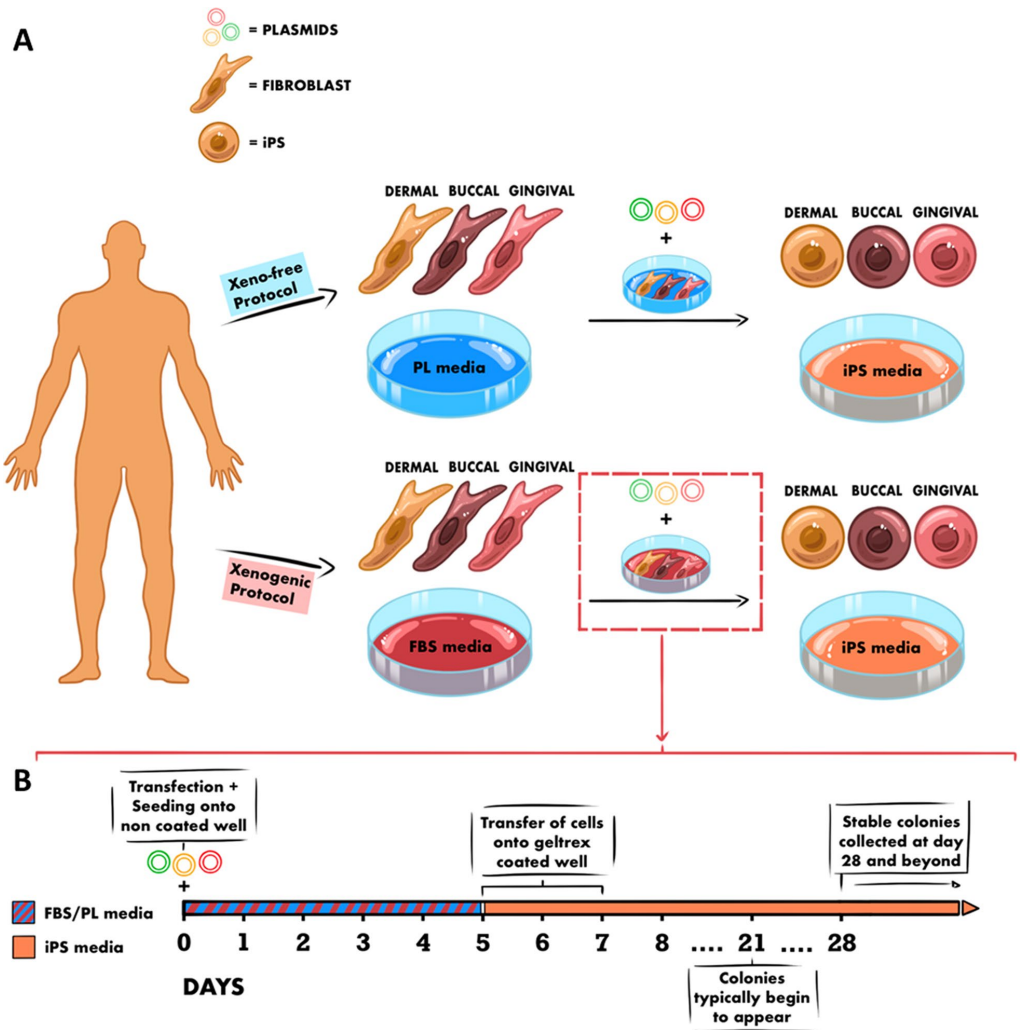
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Fig. 2 **A** Diagram illustrating the xeno/xeno-free generation of iPS, via episomal plasmid transfection, from different sources of matched fibroblasts. **B** Detailed illustration of the transfection/reprogramming procedure. This figure was created using Procreate 5.2 on iOS software (iPS: induced pluripotent stem cells, FBS: fetal bovine serum, PL: platelet lysate)
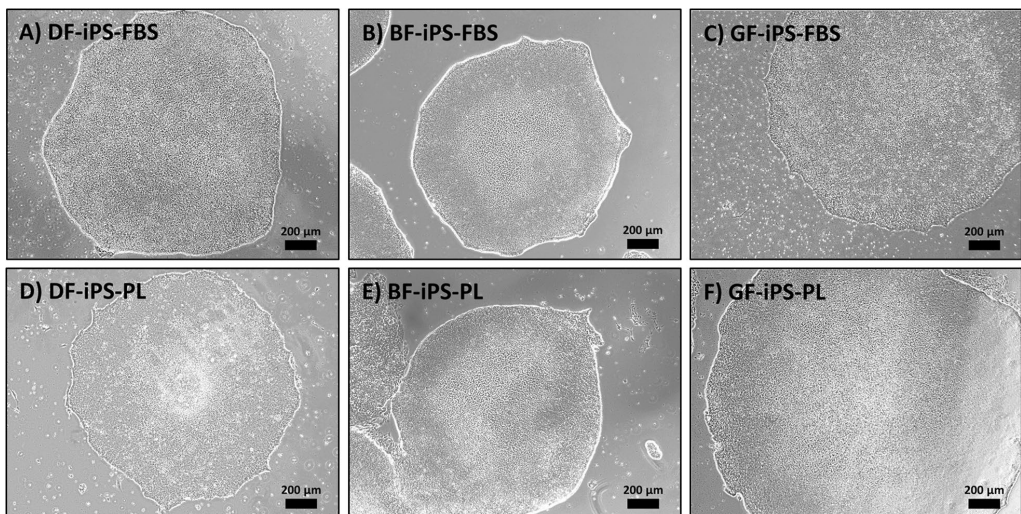
Mesoderm

Induced iPS-PL expressed *MESP1* at a significantly higher rate than their matched FBS, except in the case of DF-iPS where the expression was comparable. Expression of *OSR1* was comparable between the induced iPS-FBS and iPS-PL. Induced iPS-FBS revealed significantly higher expression of *HOPX* than their

respective matched iPS-PL, except in the case of the BF-iPS where the iPS-PL displayed significantly higher expression than the iPS-FBS.

Within the induced iPS-FBS, expression of mesoderm markers was generally comparable throughout. Within the induced iPS-PL, *MESP1* expression was higher in the oral iPS than the DF-iPS, while *HOPX* expression by the DF-iPS and BF-iPS was significantly higher than

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Table 1 Data on the reprogramming of fibroblasts expanded in FBS/PL supplemented media

Fibroblasts	Success rate (number of reprogramming procedures attempted)		Successful reprogramming		First day of iPS colony collection	
DONOR 1	**FBS**	**PL**	**FBS**	**PL**	**FBS**	**PL**
DERMAL	100% (1)	33% (3)	Yes	Yes	32	33
BUCCAL	100% (1)	50% (2)	Yes	Yes	34	35
GINGIVAL	100% (1)	11% (9)	Yes	Yes	32	39
Fibroblasts	**Success rate (number of reprogramming procedures attempted)**		**Successful reprogramming**		**First day of iPS colony collection**	
DONOR 2	**FBS**	**PL**	**FBS**	**PL**	**FBS**	**PL**
DERMAL	50% (2)	33% (3)	Yes	Yes	28	31
BUCCAL	11% (9)	0% (9)	Yes	N/A	77	N/A
GINGIVAL	20% (5)	25% (4)	Yes	Yes	31	62

Fig. 3 Representative light microscopy images demonstrating the morphology of the iPS from different sources expanded in FBS (A–C) and PL (D–F). Scale bar: 200 µm (DF: dermal fibroblasts, BF: buccal fibroblasts, GF: gingival fibroblasts, iPS: induced pluripotent stem cells, FBS: fetal bovine serum, PL: platelet lysate)

the GF-iPS. The expression of *OSR1* was comparable in the iPS-PL.

Endoderm

Induced iPS-FBS expressed significantly higher levels of *GATA4* than their matched iPS-PL, except in the case of the DF-iPS where expression was slightly higher in the iPS-PL. Within the induced iPS-FBS, the BF-iPS expressed higher level of *GATA4* than both the DF-iPS and the GF-iPS (significantly higher), with the DF-iPS expressing higher levels than the GF-iPS. In the PL group,

the DF-iPS showed higher *GATA4* expression than both the BF-iPS and the GF-iPS (significantly higher), with the BF-iPS expressing higher levels than the GF-iPS.

Ectoderm

Induced iPS-FBS expressed higher levels of *PAX6* than their matched iPS-PL (significantly higher in the case of the GF-iPS), except in the case of BF-iPS where expression was comparable. *RAX* expression was higher in the iPS-FBS than their matched iPS-PL (significantly higher in the case of GF-iPS), except in the case of the
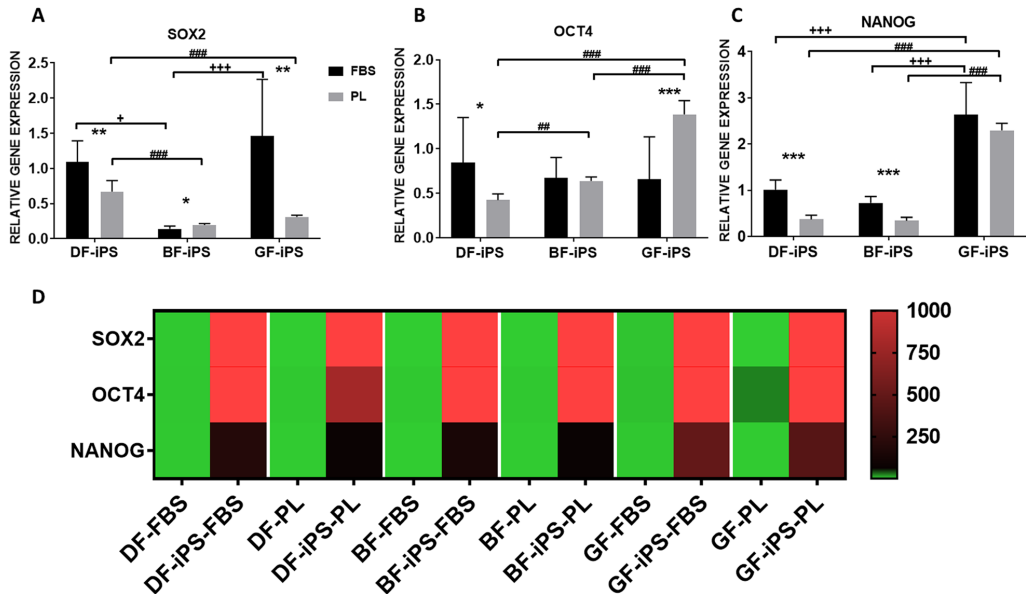
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Fig. 4 Relative gene expression of **A** *SOX2* **B** *OCT4* and **C)** *NANOG* by iPS in FBS/PL. Expression is presented relative to the DF-iPS-FBS group ± SD. **D** Heatmap analysis of the gene expression of *SOX2*, *OCT4* and *NANOG* by the fibroblasts in FBS/PL and their resultant iPS. Expression presented relative to the DF-FBS group. Independent samples t-test and one-way ANOVA were used to determine statistical significance ($p < 0.05$). (*) represents significance between iPS, from the same source, grown in FBS to those grown in PL. (+) represents significance between the iPS-FBS. (#) represents significance between the iPS-PL (DF: dermal fibroblasts, BF: buccal fibroblasts, GF: gingival fibroblasts, iPS: induced pluripotent stem cells, FBS: fetal bovine serum, PL: platelet lysate)

DF-iPS where the iPS-PL displayed significantly higher expression levels.

Within the iPS-FBS, *PAX6* expression was comparable. The DF-iPS and GF-iPS showed comparable expression levels and significantly higher levels than the BF-iPS. Within the induced iPS-PL, *PAX6* expression by the GF-iPS was downregulated compared to the DF-iPS and BF-iPS, which showed comparable expression. *RAX* expression was highest in the DF-iPS, followed by the BF-iPS.

Genetic stability of iPS

Chromosomal analysis revealed multiple amplifications and deletions within the genome of the iPS (Fig. 7). The iPS-FBS showed amplifications in chromosome 1, 5, 13 and X, and deletions in chromosomes 4, 11 and 16. The iPS-PL showed amplifications in chromosomes 1, 5, 6, 8, 13 and 17, and deletions in chromosomes 7, 11 and 16. A detailed genomic analysis of the iPS, including size and locations of the CNVs, can be found in Additional file 2.

Discussion

To comply with GMP guidelines, cells must be cultured in xeno-free conditions prior to their use for iPS generation. To our knowledge, only a handful of studies have generated iPS from entirely xeno-free conditions [45–48]. Additionally, in most cases, no effort was made to compare iPS generated via xeno-free protocols to those generated in xenogenic ones. This paper is strengthened by the standardized conditions that the cells were subjected to throughout the entirety of the project. Donor-matched fibroblasts were cultured in xeno-free PL supplemented media and separately in xenogenic FBS supplemented media, simultaneously, from the time of isolation up until 1 week post-transfection. In addition, the fibroblasts were all transfected with the same cocktail of transcription factors, via the same method of delivery and in the same laboratory. These standardized conditions allow for efficient comparisons between xenogenic and xeno-free fibroblasts in the generation of iPS.

In this study, the reprogramming of donor-matched DF, BF and GF from two donors was attempted. The attempts, however, were not always successful, as
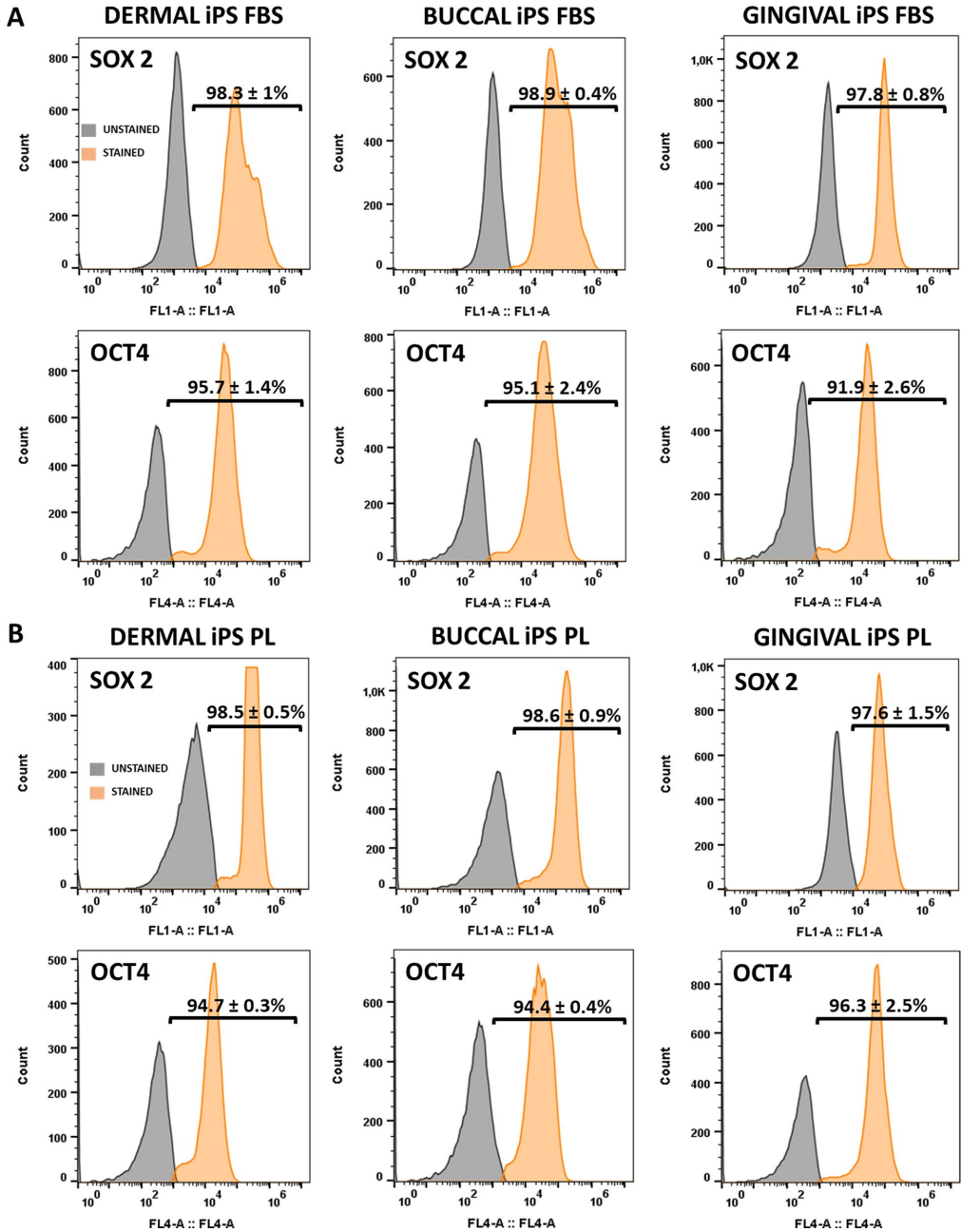
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Fig. 5 Flow cytometric analysis of **A** iPS-FBS and **B** iPS-PL showing detection of intracellular pluripotent markers SOX2 and OCT4 (percentage averages ± standard deviation) (iPS: induced pluripotent stem cells, FBS: fetal bovine serum, PL: platelet lysate)

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presented in Table 1. Chow et al. also reported similar difficulties in obtaining iPS colonies from adult canine DF [51]. Such difficulties generally represent one of the major drawbacks associated with iPS production, as the efficiency of reprogramming somatic cells is deemed to be quite low, with efficiency levels as low as 0.0006% [22]. Several theories have been postulated in attempts to explain why only a small portion of transduced cells gain pluripotency. The general consensus is that cell reprogramming comprises two main phases: a primary stochastic phase and a secondary more deterministic phase [20]. Completion of both phases appears to be a rare event for most cells, hence the low reprogramming efficiency levels. With that being said, it is reasonable to expect that in some cases the reprogramming cycle would fail altogether. From the data presented here, it seems that a transition to a PL culturing protocol was less supportive of fibroblast reprogramming to iPS. A previous report from Sung et al. corroborates our results, where in their study, PL was also found to be less efficient at inducing cell reprogramming of human amniotic fluid stem cells. Reprogramming efficiency was also found to be significantly higher in the cells cultured in FBS supplemented media [46]. The literature has shown that PL culturing protocols do indeed affect the behavior of other cell types as well. For instance, PL has been reported to increase fibroblast proliferation rates compared to FBS [52]. In MSC, both disruption and maintenance of the undifferentiated cell state have been reported to be induced by PL [7, 53–55]. These conflicting reports could be attributed to the heterogeneity that exists between PL batches, due to the pooling of the blood derivatives from multiple donors [56]. Such PL-associated changes may likely have an effect on the behavior of fibroblasts and in turn affect their ability to differentiate to iPS.

When comparing cells from multiple individuals, despite the cell type being the same, donor variability must be accounted for. Cells obtained from different individuals tend to behave differently, both morphologically and functionally [57]. Similarly, inter-donor disparities, among other factors, may lead to variations among iPS [50, 58, 59]. For instance, cells obtained from the elderly are associated with an increased risk of iPS abnormalities and a decrease in reprogramming efficiency [60–62]. The results from this study clearly show different cellular

responses, between the two donors, to the transfection procedure. For example, the BF from D1 (FBS/PL—100% success rate) were highly susceptible to reprogramming compared to the BF from D2 (FBS—33% success rate, PL—0% success rate). Besides donor variation, such differences may also be attributed to CNVs that might be acquired during the fibroblast reprogramming process [63]. These findings advocate for further investigations on the effect of donor variability on cellular reprogramming/iPS generation.

Certain elements must be considered when attempting to select the optimal cell source for reprogramming purposes, including invasiveness of the surgical procedure, ease of isolation and maintenance, and susceptibility to the reprogramming process [64]. Different somatic cell types have displayed varying results in terms of reprogramming susceptibility. Studies have revealed, for example, that keratinocytes are more easily reprogrammed than fibroblasts and that dental pulp stem cells yield more iPS colonies than bone marrow MSC [30, 33]. This disparity in susceptibility makes selection of the ideal cell source for reprogramming quite difficult. In this study, we found that DF are generally easier to reprogram than BF and GF. Yan et al. also reported similar difficulties when attempting to reprogram GF, with various transfection protocols yielding no iPS colonies [33]. It is not clear in their study, however, how many attempts were made to generate iPS from the GF. As it is with the GF in this study, it might be that continued attempts would have eventually led to the development of pluripotent colonies. The exact reasons as to why oral fibroblasts reprogram less efficiently than DF are unclear. However, inherent phenotypical differences between the fibroblasts are likely to play a role in reprogramming efficiency [65, 66]. Further investigation is required to determine the correlation and effect such innate characteristics have on the reprogramming process.

Due to the BF-PL from D2 not yielding any iPS colonies, the focus was shifted to the results obtained from the analysis of D1. This allows for a more efficient comparison of the effect that different sources and culture conditions have on iPS generation. According to the literature, there are different levels of pluripotency, and cells should fulfill certain criteria at each level before being deemed as pluripotent [67, 68]. These criteria

(See figure on next page.)

Fig. 6 Relative gene expression of the **A–C** mesoderm, **D** endoderm and **E, F** ectoderm markers by the iPS following directed differentiation toward the three lineages. **G** Heatmap analysis of the gene expression of trilineage markers by the iPS before and after directed (induced) differentiation. Expression is presented relative to the uninduced DF-iPS-FBS group ± SD (uninduced iPS not shown on graphs). Independent samples t-test and one-way ANOVA were used to determine statistical significance ($p < 0.05$). (*) represents significance between the induced iPS, from the same source, grown in FBS to those grown in PL. (+) represents significance between the induced iPS-FBS. (#) represents significance between the induced iPS-PL (DF: dermal fibroblasts, BF: buccal fibroblasts, GF: gingival fibroblasts, iPS: induced pluripotent stem cells, FBS: fetal bovine serum, PL: platelet lysate, ENDO: endoderm, MESO: mesoderm, ECTO: ectoderm)
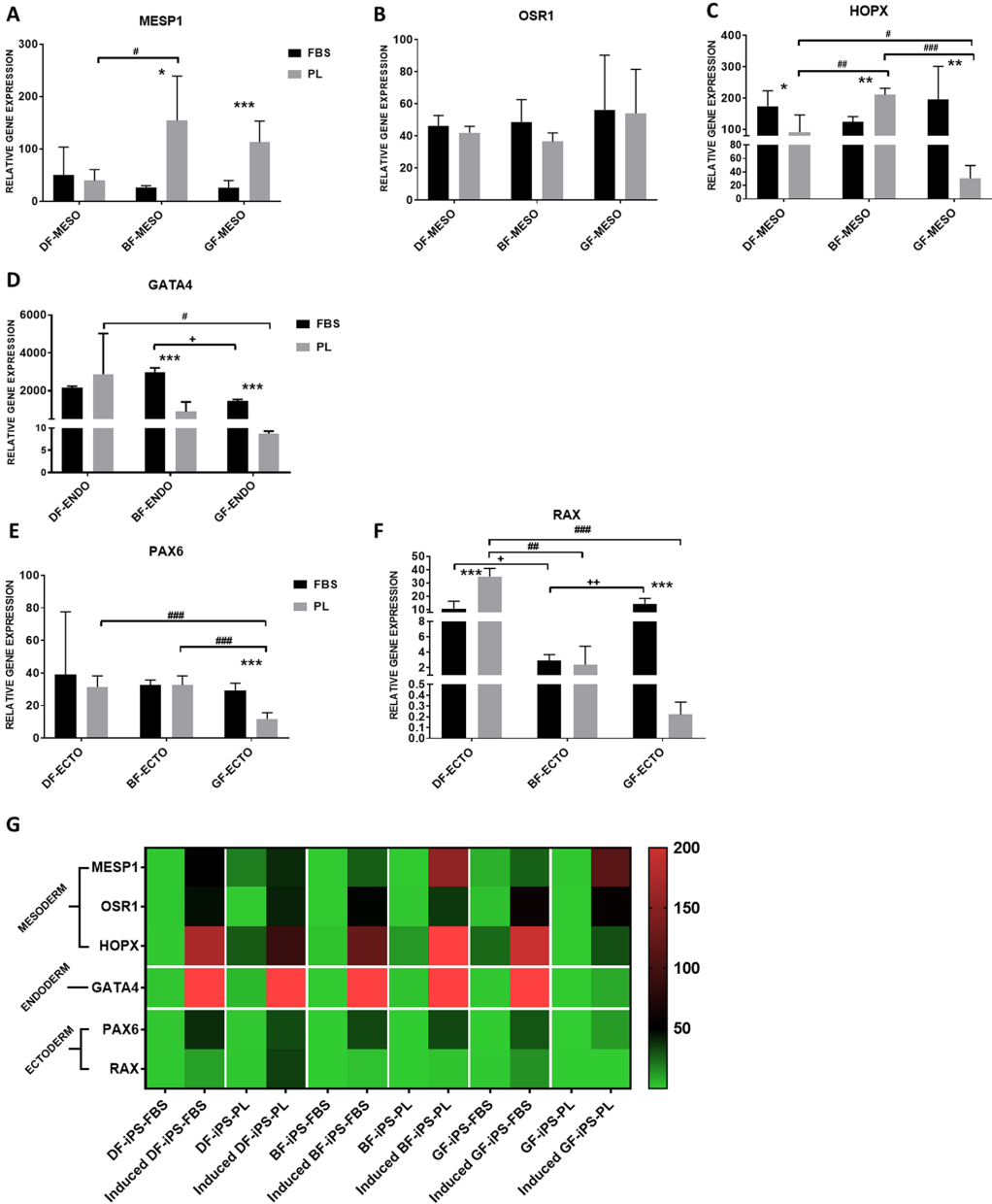
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Fig. 6 (See legend on previous page.)

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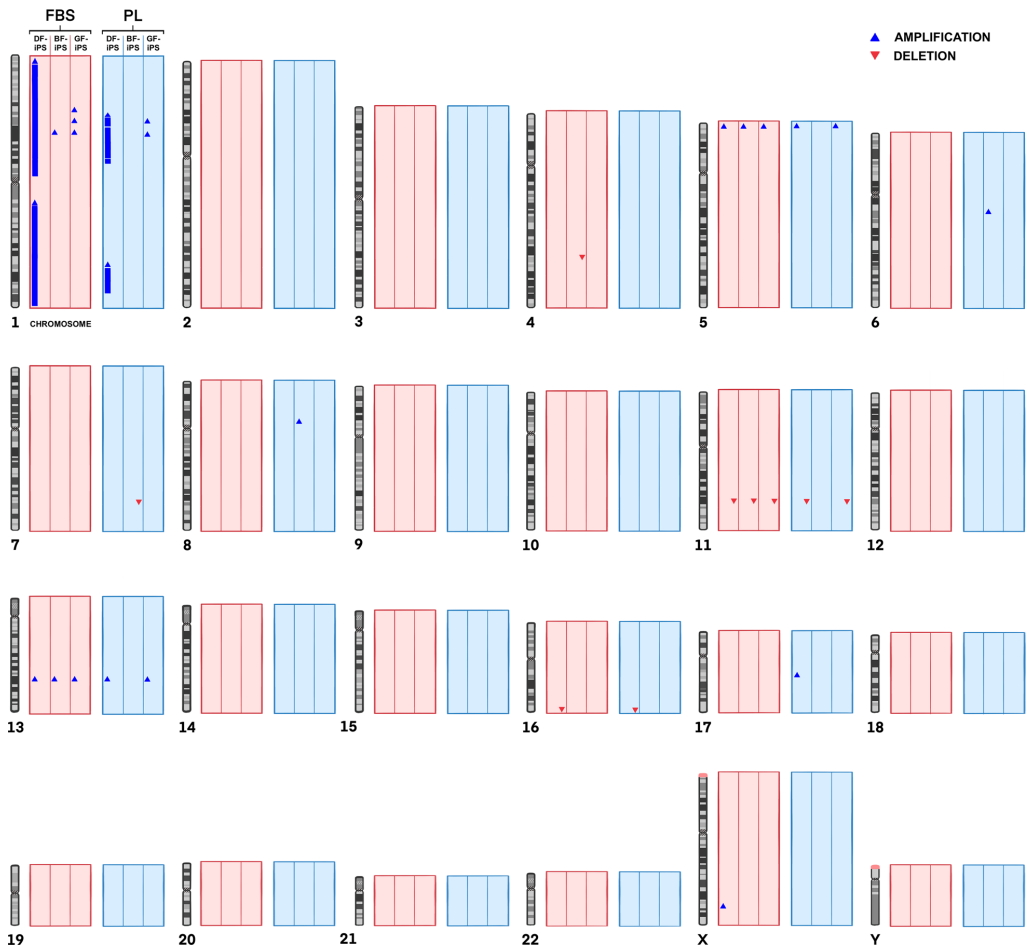
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Fig. 7 Representative* figure displaying the chromosomal CNVs of the different iPS-FBS and iPS-PL. The arrows/bars represent the gain (blue) or loss (red) of a chromosomal region. *Intended only as a representative figure and not for displaying exact locations of each CNV (DF: dermal fibroblasts, BF: buccal fibroblasts, GF: gingival fibroblasts, iPS: induced pluripotent stem cells, FBS: fetal bovine serum, PL: platelet lysate)

include cell/colony morphology, expression of pluripotency markers and ability to differentiate into the three primary germ layers. Once the iPS in this study were established, we analyzed their pluripotency at a cellular, molecular and functional level. At a cellular level, all the cells developed a similar colony morphology to ESC, and although not identical, they fall in the category of stable iPS colony morphology [50, 69]. At a molecular level, they expressed genes (*SOX2*, *OCT4*, *NANOG)* and proteins (OCT4, SOX2) which play a major role in inducing and maintaining the

pluripotency of ESC and iPS [68]. They also displayed functional pluripotency and expressed markers associated with mesoderm (*MESP1*, *OSR1*, *HOPX*), endoderm (*GATA4*) and ectoderm (*PAX6*, *RAX*) lineages following directed differentiation. These findings demonstrate that these iPS are pluripotent and possess ESC-like characteristics. Despite the decrease in reprogramming efficiency, moving to a xeno-free protocol does not seem to have any detrimental effect on the cells after successful induction of pluripotency, as no major differences were seen between the genotype/phenotype

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of the iPS-PL and iPS-FBS [46]. At gene level, iPS-PL generally expressed slightly lower levels of the pluripotent markers than iPS-FBS; however, differences were insignificant. Furthermore, these differences did not seem to translate at protein level, with flow cytometry analysis revealing comparable detection of pluripotent proteins by both sets of iPS. The ability of iPS to differentiate to the three primary germ layers does not appear to be negatively affected by the use of PL, with the iPS-PL and iPS-FBS expressing comparable levels of the trilineage markers upon directed differentiation. The differentiated GF-iPS-PL expressed the markers *GATA4* and *RAX* significantly higher than the non-differentiated iPS. However, this expression was much less than the rest of the differentiated iPS, including its xenogenic counterpart. The reason for this relatively low expression is unclear. Perhaps the xeno-free protocol caused these particular cells to differentiate much slower toward endoderm and ectoderm lineages, and an increase in the duration of differentiation might result in similar expression levels to the other iPS.

With the introduction of new supplements for cell culturing protocols, it is important to ensure that no major alterations occur within the cell genome as a result of supplementation. When assessing the genetic state of human pluripotent cells, the literature shows that chromosomes 1, 12, 17, 20 and X are generally the most affected [26, 27]. Interestingly, a different set of chromosomes were more commonly affected within both iPS groups, specifically chromosomes 1, 5, 11 and 13. CNVs affecting these specific chromosomes were seen in all the iPS apart from BF-iPS-PL. Overall, both sets of iPS revealed a relatively low amount of CNVs, with the exception of the DF-iPS in both conditions, which show duplications of several segments in chromosome 1. Somatic mosaicism in the culture of fibroblasts has been shown to cause most of the genetic variation in their resultant iPS [26]. Similarly, Abyzov et al. [70] revealed in a study involving dermal fibroblasts/iPS, that 50% of the CNVs found in the iPS were present in their parental fibroblasts. This, however, is not the case with the DF-iPS in this study, as the genetic analysis revealed no CNVs in chromosome 1 of their parent fibroblasts. Hence, this particular genetic change is likely a result of the reprogramming process, or cell culture and propagation [71]. Despite their being links between aberrations in chromosome 1 to tumor formation [72], the majority of the evidence points to the harmlessness of chromosomal abnormalities in iPS. Ultimately, these genetic alterations are a common occurrence, as human pluripotent cells are often genetically unstable. Furthermore, both genomically normal and abnormal iPS can lead to teratoma formation, and

there is little evidence linking the genomic abnormalities with tumorigenesis [26, 27, 63].

Analyzing the effect of cell source on iPS genotype/phenotype revealed only minor differences within each group. No particular trend was observed, however, and thus, none of the variations could be directly attributed to differences in cell source. Ultimately, these results confirm the ability to generate safe iPS from oral and dermal fibroblasts in xeno-free conditions, with quality comparable to those generated in FBS. This should allow for a smooth transition to utilizing xeno-free oral iPS for research at both the preclinical and clinical stage.

Conclusion

For the purposes of future stem cell research and clinical translation, generating iPS in xeno-free conditions serves as a favorable strategy. When compared to FBS, the use of PL in culture media appears to lower reprogramming efficiency. Nevertheless, xeno-free dermal, buccal and gingival fibroblasts can successfully generate iPS similar to their xenogenic counterparts. The nature of fibroblast source and expansion conditions appear to have little effect on iPS genotype/phenotype. Despite having the advantage of rapid healing with minimal scar formation, oral fibroblasts proved to be more difficult to reprogram than dermal fibroblasts. Transitioning to xeno-free oral fibroblasts for generating iPS looks to be a promising approach; however, the issue of low reprogramming efficiency must be addressed in order to boost cost-effectiveness for future research and clinical use.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13287-023-03403-7.

Additional file 1. Overview of primers used for gene expression analysis

Additional file 2. CNV analysis data

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Availability of data and materials
Additional data can be made available by the authors upon request. Chromosome microarray analysis data archived and shared, according to SN data policy, to NCBI's dbVar public repository (accession: nstd231) at https://www.ncbi.nlm.nih.gov/dbvar/studies/nstd231/.

Declarations

Ethics approval and consent to participate
Ethical approval for the collection and use of the human tissue samples was granted by the Ethical Committee for Medical and Health Related Research in West Norway (Approval number and date of approval: REK 80005, 14.06.2021). Tissue samples were collected from two healthy voluntary donors after acquiring informed consent. The title of the approved project is as follows: "Dual targeted strategy for cancer therapy and bone regeneration by use of nanodiamond loaded mesenchymal stem cells derived from adult fibroblasts."

Consent for publication
Not applicable.

Competing interests
The authors confirm and declare that there are no known conflicts of interest associated with this publication.

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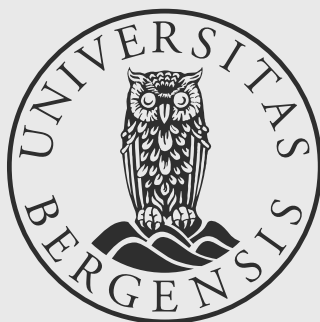
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Paper III

Osteogenic differentiation of induced pluripotent stem cells derived from dermal and oral tissues in xeno-free medium

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